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<b>(54) Title:</b> DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR INDUCING AN ANTI-TUMOR RESPONSE <b>(57) Abstract</b> <p>The invention provides dendritic-like cell/tumor cell hybridomas and pluralities of dendritic-like cell/tumor cell hybrids that confer tumor resistance <i>in vivo</i>. The hybrids and hybridomas are generated by the fusion of tumor cells with dendritic-like cells. For instance, immortal tumor cells from an autologous tumor cell line can be fused with autologous or HLA-matched allogeneic dendritic-like cells. Autologous tumor cell lines can be derived from primary tumors and from their metastases. Alternatively, immortal dendritic-like cells from an autologous or allogeneic HLA-matched dendritic-like cell line can be fused with autologous tumor cells. Autologous dendritic-like cell lines can be prepared from various sources such as peripheral blood and bone marrow. Dendritic-like cell/tumor cell hybridomas and pluralities of hybrids can be directly infused for active immunization of cancer patients against their residual tumor cells. The hybridomas and hybrids can also be used for the <i>in vitro</i> activation of autologous immune cells before their reinfusion into the patient for passive immunization against the tumor cells.</p>		

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DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR  
INDUCING AN ANTI-TUMOR RESPONSE

Technical Field

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This invention is in the field of immunotherapy for the treatment of cancer. Specifically, the invention provides a hybridoma consisting of a fused tumor cell and a dendritic-like cell, which hybridoma is capable of inducing an anti-tumor response in vivo when administered to a subject in need of anti-tumor treatment.

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Background of the Invention

The Immune Response

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The introduction of pathogens such as bacteria, parasites or viruses into a mammal elicits a response contributing to the specific elimination of the foreign organism. Foreign material is referred to as antigen, and the specific response is called the immune response. The immune response starts with the recognition of the antigen by a lymphocyte, proceeds with the elaboration of specific cellular and humoral effectors and ends with the elimination of the antigen by the specific effectors. The specific effectors are essentially T lymphocytes and antibodies, mediating cellular and humoral immune responses, respectively. The present invention relates to the initiation of a cellular immune response. The initiation of a cellular immune response starts with the recognition of an antigen on the surface of an antigen-presenting cell (APC).

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Antigen Recognition by T-Lymphocytes

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Cellular antigen recognition is operated by a subset of lymphocytes called T-lymphocytes. T-lymphocytes include two major functional subsets. They are T-helper lymphocytes (TH), that usually express the CD4 surface marker, and cytotoxic T-lymphocytes (CTL), that usually express the CD8 surface marker. Both T-cell subsets express an antigen receptor that can recognize a given peptide antigen. The peptide needs to be associated with a major histocompatibility molecule (MHC) expressed on the surface of the APC, a phenomenon known as MHC restriction. T-cells bearing the CD4 surface marker recognize peptides associated with MHC class II molecules,

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whereas T-cells bearing the CD8 surface marker recognize peptides associated with MHC class I molecules.

Since the T-cell antigen receptor can only recognize peptides associated with MHC molecules at the surface of an APC, cellular proteins need to be processed into such peptides and transported with MHC molecules to the cell surface. This is referred to as antigen processing. Exogenous proteins, phagocytosed by the APC, are broken down into peptides that are transported on MHC class II molecules to the cell surface, where they can be recognized by CD4<sup>+</sup> T-cells. In contrast, endogenous proteins, synthesized by the APC, are also broken down into peptides, but the latter are transported on MHC class I molecules to the cell surface, where they can be recognized by CD8<sup>+</sup> T-cells.

When a T-cell binds through its antigen receptor to its cognate peptide-MHC complex on an APC, the binding generates a first signal from the T-cell membrane towards its nucleus. However, this first signal is insufficient to activate the T-cell, at least as measured by the induction of IL-2 synthesis and secretion. Activation only occurs if a second signal or costimulatory signal is generated by the binding of other APC surface molecules to their appropriate receptors on the T-cell surface. The best known costimulatory molecules identified to date on APC are B7-1 (Razi-Wolf et al, 1993, Proc. Natl. Acad. Sci. USA 90: 11182-1186) and B7-2 (Hathcock et al, 1993, Science 262: 905-907); both bind to the CD28/CTLA4 counter-receptor on T-lymphocytes. The capacity to present peptide antigens together with costimulatory molecules in such a way as to activate T-cells is hereafter referred to as antigen presentation. Only APCs have the capacity to present antigens to CD4<sup>+</sup> (predominantly TH) and CD8<sup>+</sup> (predominantly CTL) T-cells, leading to the development of humoral and cellular immune responses.

#### T-lymphocyte Activation by Antigen-Presenting Cells

APCs are heterogeneous in their cell lineage and functional performance. They include distinct cell types such as B lymphocytes, T lymphocytes, monocytes/

macrophages and dendritic cells from myeloid origin. All these cells are bone marrow-derived cells, that need to mature and to be activated in order to function efficiently as APCs.

The functional performances of APCs rely critically upon the nature and state of maturation of the cells included in purified or enriched APC preparations. The latter vary with the tissue of origin and method of purification. In an operational way, we call dendritic-like cells (DLCs) ) all non-B cells present in purified or enriched preparations of dendritic cells. These cells all share some morphological, physical or biochemical characteristics with dendritic cells, leading to their co-purification with dendritic cells. Therefore, the term DLCs refers hereafter not only to dendritic cells of myeloid origin, but also to monocytes, T-lymphocytes and other non-Bcells present in enriched or purified dendritic cell preparations. In mice, the spleen is very often used as a source of DLCs (reviewed by Steinman, 1991, Annu. Rev Immunol 9: 271-296). However, mouse DLCs have also been generated by in vitro culture from bone marrow progenitors in the presence of cytokines (Inaba et al, 1992, J. Exp. Med. 176: 1693-1702). In humans, blood or bone marrow are the usual sources of DLCs, that are used either immediately or more often after culture in the presence of cytokines. Several protocols of purification and in vitro culture have been published (reviewed in Young and Inaba, 1996, J. Exp. Med 183: 7-11), and patent applications have been filed for some of them (WO 93/20185, by Steinman R., Inaba K. Schuler G WO 93/20186, by Banchereau J and Caux C; WO 94/02156 by Engleman E., Markowicz S and Metha A; WO 95/28479 by Brugger W and colleagues of Mertelsmann R.).

#### T-Lymphocyte Activation by Tumor Cells

There is increasing evidence that tumor cells do not usually function as APCs (reviewed by Young and Inaba, 1996, J. Exp. Med 183: 7-11). Although some tumor cells are capable of delivering an antigen-specific signal to T cells, they may not provide the costimulatory signals which are necessary for the full activation of T-cells and thereby fail to induce an efficient anti-tumor immune response. In order to compensate for this inefficient induction of an anti-tumor immune response, different

approaches have been tried in experimental animals (reviewed by Grabbe et al, 1995, Immunology Today 16: 117-121).

In one such approach, tumor cells were genetically engineered to express one or more molecules known to be involved in antigen presentation on APC. To date, efficient in vivo results from this approach were obtained with tumor cells co-expressing MHC class I, MHC class II and B7-1 molecules, suggesting that the successful immunotherapy was linked to the activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. For example, Basker et al, 1995, J. Exp. Med 181:619-629 engineered mouse fibrosarcoma cells, that naturally express MHC class I molecules, to express in addition MHC class II and B7-1 molecules; the injection of these modified tumor cells was sufficient to cure syngeneic mice carrying large established tumors. It should be noted that tumor cells expressing MHC class I but not MHC class II molecules and transduced with the B7-1 costimulator also induced an in vivo anti-tumor immune response, and that the latter depended upon the activation of CD8<sup>+</sup>, but not CD4<sup>+</sup> T cells (Ramarathinam et al, 1994, J. Exp. Med. 179: 1205-1214). The disadvantage of this approach lies in the genetic engineering of the tumor cells, a technique that usually involves the use of viral vectors for efficient gene transfer. Viral vectors are not totally safe for the treatment of human patients. The main reason is that they can recombine both in vitro and in vivo, which may lead to the production of novel wild type viruses of unpredictable pathogenicity. This limitation stimulated the development of alternative methods of efficient gene transfer, such as the one recently described by Birnstiel and colleagues (WO 94/21808).

In another approach, APCs were loaded with a source of tumor antigens. Amongst the APC tested for such a purpose, DLCs appeared to be the most efficient. To date, it is clear that DLCs pulsed with tumor cell lysates (Knight et al, 1985, Proc. Natl. Acad. Sci. USA 82: 4495-4497), with a purified tumor-associated protein (Flamand et al, 1994, Eur. J. Immunol 24: 605 - 610; Paglia et al, 1996, J. Exp. Med. 183: 317-322) or with tumor-associated peptides (Ossevoort et al, 1995, J. Immunotherapy 18: 86-94; Mayordomo et al, 1995, Nature Medicine, 1: 1297-1302)

can efficiently induce an anti-tumor immune response in vivo. There are, however, disadvantages to this approach. Tumor cell lysates or fractions thereof are relatively easy to prepare, but the loading of DLCs with such crude preparations could, at least theoretically, induce adverse auto-immune reactions in the host. Similar secondary effects could be induced by DLCs loaded with all the peptides eluted from tumor cells, as described by Zitvogel et al, J. Exp. Med. 1996, 183: 87-97. The latter risk is reduced by pulsing DLCs with purified, tumor-specific antigens or peptides. However, there are very few known tumor-specific antigens, and in addition, their production and purification are both labor-intensive and expensive.

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In a recent approach, a tumor cell and one sort of APC, namely a B-lymphocyte, were united into a single cell by somatic cell fusion (Guo et al, 1994, Science 263: 518-520). Guo et al. fused a rat hepatoma cell line with in vivo-activated B lymphocytes, and showed that some of the resulting B cell/tumor cell hybridomas induced tumor-resistance in syngeneic rats and also cured the animals of a small pre-established tumor. The selected hybridomas expressed MHC class II restriction elements and B7 costimulatory molecules, which strongly suggested that the immunotherapy worked through the activation of CD4<sup>+</sup> TH cells. When compared to the two previous approaches, this third approach has the general advantages of somatic cell fusion, namely, it brings together not only the known tumor antigens and known costimulators of activated B-cells, but possibly some as yet unknown molecules carrying out these functions. When compared to the genetic engineering of tumor cells, this cellular engineering does not require the identification of the genes encoding costimulatory molecules, nor their transfer into tumor cells. Similarly, when compared to the pulsing of APC with purified tumor-specific antigens, somatic cell fusion does not require the identification of genes encoding tumor-specific antigens, nor the production and purification of the corresponding recombinant proteins. However, in its present description, this approach is inapplicable to human cancer patients, because it involves the use of in vivo-activated B-cells as fusion partners of the tumor cells. In vivo-activated B-cells were recovered from the spleen fourteen days after immunization

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with soluble antigen in complete Freund's adjuvant, which cannot be used in humans. In addition, if immunizations are done without Freund's adjuvant, the outcome of an in vivo activation of B-cells remains unpredictable in individual animals, and it is expected to be unpredictable in individual human patients. Finally, the selection of the hybridomas is quite labor-intensive. It required the preparation, absorption and characterization of tumor-specific polyclonal antisera, that were used to select the cells expressing surface markers of the tumor parent; this first selection was then followed by a second selection of cells expressing surface markers of the in vivo-activated B-cell parent.

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What is really needed is a method to harness the ability of DLCs to elicit an anti-tumor response, so that the immune system of a subject can mount a rejection of the tumor cells. In addition, this method should be transposable to human cancer patients.

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#### Summary of the Invention

The present invention provides DLC/tumor cell hybridomas and a plurality of DLC/tumor cell hybrids for use in the treatment of cancers. The hybridomas and hybrids of the invention are capable of inducing an anti-tumor response when administered to the subject, in vivo.

A DLC/tumor cell hybridoma of the invention is produced by first providing a sample of the specific tumor against which an immune response is needed. In one embodiment of the invention, an immortal cell line is derived from the tumor sample, and then the tumor cells are fused with DLCs. Preferably, autologous DLCs from the subject are used, but matched HLA-compatible DLCs may also be used as fusion partners. Once the DLCs are fused with the tumor cells, selection is carried out. In this embodiment, hybridomas which exhibit DLCs characteristics are selected, their immortality being necessarily contributed by fusion with the tumor cell. In a second embodiment of the invention, an established immortal human tumor cell line is

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provided which expresses at least one of the tumor-associated antigens of the patient's tumor cells. Cells from the tumor cell line are fused with autologous or HLA-compatible allogeneic DLCs to form hybridomas which are then selected for retention of DLC characteristics. In a third embodiment of the invention, an immortal DLC line is established, and then DLCs of this line are fused with the patient's tumor cells from primary culture. The resulting hybridomas are selected for retention of DLC characteristics as well as expression of at least one tumor-associated antigen of the patient's tumor cells.

10 In other embodiments of the invention, tumor cells are fused with DLCs, and the resulting plurality of hybrids is used directly for treatment, without selection.

The DLC/tumor cell hybridoma, or plurality of hybrids, is administered to the subject to induce an immune response against residual tumor cells in the subject's circulation or organs. Alternatively, the hybridoma or plurality of hybrids is co-cultivated in vitro with immune cells from the subject in order to activate against the tumor cell; the activated immune cells are then returned to the subject.

#### Brief Description of the Drawings

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Figure 1. Scanning electron microscopy of parent cells and of two murine DLC/tumor cell hybridomas (x 4,000). The figure illustrates the "tumor-like" and "dendritic-like" characteristics of two DLC/tumor cell hybridomas. Hybridoma HY1 (Fig. 1c) resembles more the parent P815\* tumor cell (Fig. 1a) than the parent dendritic cell (Fig. 1b), whereas hybridoma HY41 (Fig. 1d) resembles more the dendritic cell (Fig. 1b) than the P815\* tumor cell (Fig. 1a). It is the "dendritic-like" hybridoma HY41 that was selected for in vivo experiments.

30 Figure 2a-e. FACS analysis of DLC/tumor cell hybridomas HY41 and HY62, showing the expression of CD3 and the TCR V- $\beta$ 8 domain by the CD3-positive subclones

(HY41 CD3+ and HY62 CD3+); the CD3-negative subclones of these hybridomas (HY41 CD3- and HY62 CD3-) as well as the parent P815\* tumor cells fail to express the TCR V- $\beta$ 8 domain.

- 5 Figure 3. Ethidium bromide-stained gel electrophoresis of Polymerase Chain Reaction products obtained with mouse genomic DNA, using TCR V- $\beta$ 8 and C $\beta$  primers. A rearranged TCR $\beta$  gene fragment was amplified from genomic DNA of a mouse T-cell hybridoma (T), as well as from the HY41 (41) and HY62 (62) DLC/tumor cell hybridomas; no rearranged TCR $\beta$  fragment was amplified from DNA of P815\* tumor cells (P) and spleen cells (S), used as negative controls.
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Figure 4. Survival curves of immunocompetent and immunocompromised (i.e. irradiated) DBA/2 mice after ip inoculation with  $5 \times 10^5$  syngeneic hybridoma cells HY41 or with the same number of parental P815\* tumor cells.

- 15 Key: ○ P815 in normal mice (n = 10)  
○ P815 in irradiated mice (n = 10)  
△ HY 41 in normal mice (n = 12)  
△ HY 41 in irradiated mice (n = 10)

- 20 This figure shows that the "dendritic-like" hybridoma HY41 was rejected by 75% (9/12) of the immunocompetent mice, while the parent tumor was rejected, in this particular experiment, by 20% (2/10) of the animals. This difference in survival was not due to a difference in tumorigenicity, since both cell lines killed all (10/10) immunocompromised animals within four weeks of inoculation.

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Figure 5. Survival curves of naive and HY41-treated DBA/2 mice after ip inoculation with  $5 \times 10^5$  syngeneic P815\* tumor cells. Y-axis = survival (%). X-axis = weeks after inoculation.

Key: ○ normal mice (n = 9)

- 30 ▽ "HY41" - treated mice (n = 9)

This figure shows that the nine HY41-survivors (see Fig. 4) became at least partially resistant to a lethal challenge with the parental P815\* tumor cells, and that 4/9 of these animals showed complete tumor resistance for at least three months.

5 Figure 6. P 815 Targets (T). Chromium release assay on P815\* and L1210 target cells with spleen cells from individual mice. Y-axis = Cr release (%). X-axis = spleen from individual mice:effectors (E). This figure shows that the spleen cells of the four P815\*-resistant mice (see Fig. 5; individual mice nrs 5 - 8 in Fig. 6), contain a strong cytolytic activity directed against P815\* cells (Fig. 6A) but not against the irrelevant  
10 (but MHC class I-matched) L1210 tumor cells (Fig. 6B). In contrast, the spleen cells of the four naive animals (mice nrs 1-4) do not show any detectable cytolytic activity against P815\* cells (Fig. 6A). The spleen cells from individual mice (1 - 8) were cultured in vitro for five days either in the absence (x) or in the presence of P815\* stimulator cells (x + P815). Thereafter, they were used as effector cells on chromium-  
15 labelled target cells, at different effector:target (E:T) ratios.

Figure 7. Survival of mice bearing an established tumor P815\*. Y-axis = % of survival. X-axis = weeks after inoculation.

Key: ○ untreated mice (n = 10)  
20 ○ HY41 - treated mice (n = 10)  
▽ HY62 - treated mice (n = 10)  
▽ P815 - treated mice (n = 10)

Survival curves of tumor-inoculated mice treated with irradiated HY41 or HY62  
25 hybridoma cells. All mice were inoculated ip with  $2 \times 10^5$  P815\* tumor cells on day 0. The figure shows that 2 months after tumor inoculation, 6/10 and 4/10 animals treated by 4 weekly ip injections of irradiated HY41 and HY62 hybridoma cells, respectively, were alive and tumor-free. In contrast, none (0/10) of the untreated animals and only 2/10 animals treated with irradiated P815\* tumor cells were alive at that same time.

Figure 8. FACS analysis showing HLA-DR expression in human F3BG10 DLC/tumor cell hybridoma (Fig 8a) and in its subclone F3BG10-H12 (Fig 8b) before and after incubation with interferon  $\gamma$ . Before incubation with the cytokine, labeling by the anti-HLA-DR mAb (thinner line) was identical to the labeling by the isotope-matched control mAb (not shown). After 24 hours incubation with interferon  $\gamma$ , around 40% of the F3GG10 hybridoma cells and over 90% of the H12 subclone cells were specifically labeled by the anti-HLA-DR mAb (thicker line).

#### Detailed Description of the Invention

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The present invention provides DLC/tumor cell hybrids and hybridomas for activating anti-tumor responses. Although the specific procedures and methods described herein are first exemplified using a DBA/2 mouse mastocytoma cell line and DLCs isolated from syngeneic spleen, they are merely illustrative for the practice of the invention. Analogous procedures and techniques are applicable for the treatment of human subjects, as thereafter exemplified using a human osteosarcoma cell line and blood-derived DLCs. Therefore, DLC/tumor cell hybrids and hybridomas could be used to immunize human patients against their cancer. Procedures applicable to the treatment of a human subject would involve the following steps.

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(1) A sample is provided of the tumor against which an immune response is needed. Such a sample can be obtained when the primary tumor and/or its metastases are removed by surgery, as practised for example for cancers of the breast, prostate, colon, and skin. When the treatment of the cancer involves chemotherapy and/or radiotherapy rather than surgery, as practised for example for small cell lung cancer, lymphomas and leukemias, a sample of the tumor can be obtained from a metastatic site, either before treatment or after relapse. Examples of easily-accessible tumor sampling sites are the peripheral blood, bone marrow, peritoneal and pleural effusions, lymph nodes and skin.

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Tumor cells can be separated from blood or bone marrow samples, for instance, by a combination of physical, enzymatic and immunological methods. Contaminating red blood cells can be removed by osmotic lysis. Tumor cells can be concentrated by density centrifugation. Tumor cells can be separated from other cells  
5 by binding antigen on the tumor cell surface to antibody-coupled magnetic beads, which are then separated from the biological fluid by means of magnets. In negative cell selection, which may be performed prior to positive cell selection, antibodies bind to antigens that are expressed on contaminating cells, and used to deplete the biological fluids of non-tumor cells. In positive cell selection, antibodies bind to  
10 tumor-associated antigens, and this binding is used to separate tumor cells from the biological fluids. When tumor cells are separated by means of antibody-coupled magnetic beads, cells can be released from the beads by digestion of the antigen/antibody binding sites with chymopapain or by other means. The resulting separated tumor cells can re-express the tumor-associated antigen after a short time in  
15 culture. The tumor cells are expected to contribute genes encoding known and unknown tumor-associated antigens to the hybridoma of the invention.

Tumor cells can also be separated from solid tissue samples, using a combination of physical, enzymatic and immunological methods. Macroscopic peri-  
20 tumoral stromal tissue can be removed by dissection prior to reduction of the tumor to a cell suspension. Density centrifugations and antibody-mediated separations can then be performed on the cell suspension as described above.

(2) The purified tumor cells are then prepared for cell fusion. Three types of  
25 tumor partners can be prepared: (i) primary cultured tumor cells, (ii) immortal tumor cells, and (iii) drug-sensitive immortal tumor cells. Primary cultured tumor cells are purified tumor cells which have been cultured for a limited period of time in the presence of appropriate growth factors. Immortal tumor cells are permanent cell lines derived from these primary cultured tumor cells; such permanent cell lines can be  
30 obtained, for instance, after culturing the primary tumor cells for longer periods of time

in the presence of appropriate growth factors, or by transducing the primary tumor cells with immortalizing genes. Finally, drug-sensitive immortal tumor cells are permanent cell lines derived from spontaneous mutants of immortal tumor cells; these mutants are selected by culturing the immortal tumor cells in the presence of an appropriate drug. These drug-sensitive immortal tumor cells die when they are exposed to the drug to which they are sensitive. For example, 6-thioguanine was used to select the murine P815\* mastocytoma cell line described in Example 1, and 5-bromo-2'-deoxyuridine was used to select the human 143B osteosarcoma cell line described in Example 7. Both cell lines die when cultured in HAT-containing medium, as described in Examples 3 and 9.

(2a) As an alternative to step (2) a pre-established immortal human tumor cell line can be used, provided that at least one of the tumor-associated antigens from the patient's tumor cells are matched to these pre-established immortal tumor cells.

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(3) A sample is provided with a source of DLCs. Such samples include for example peripheral blood, cord blood, bone marrow, lymph or accessible lymph nodes; they may be taken from the patient or from a healthy, HLA-compatible donor. From there, two alternatives are available. Functionally-competent DLCs can be purified directly from these samples, using various methods described in the literature. Alternatively, functionally-competent DLCs can be purified after in vitro differentiation of the precursors contained in these samples, which can be done by culturing the latter in the presence of cytokines, as described hereunder.

(4) The DLCs are prepared for cell fusion, in one of the 4 following ways. (1°) Primary DLCs purified directly from blood, lymph or other tissues are maintained in culture for no longer than 24 hours, as described for mouse spleen DLCs in Example 2. (2°) Primary cultured DLCs differentiated from blood, bone marrow or other tissues are cultured for at least 7 days in the presence of cytokines, as described for human blood DLCs in Example 8 or as published by Sallusto and Lanzavecchia, 1994, J. Exp.

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Med. 179: 1109-111; Romani et al, 1994, J. Exp. Med. 180: 83-93; Mackensen et al, 1995, Blood, 86: 2699-2707. (3°) Immortal DLCs can be derived from primary-cultured DLCs, for example by adapting the method described by Paglia et al, 1993, J. Exp. Med. 178: 1893-1901. These authors immortalized neonatal mouse spleen DLCs  
5 by using a recombinant retrovirus. (4°) HAT-sensitive variants of these DLC lines can thereafter be derived by standard culture techniques, to yield drug-sensitive immortal DLCs.

(5) A tumor cell partner is then fused with a DLC partner. From there, two  
10 alternatives are available, namely to separate or not to separate the fused cells by metabolic selection. After fusion, the treated cells include a plurality of DLC/tumor cell hybrids, as well as unfused tumor cells and unfused DLCs. If no selection is applied, fused cells as well as unfused cells are used for inducing an anti-tumor immunity in vivo and/or in vitro (see § 6 ). If a metabolic selection is applied, for  
15 example by plating the treated cells in HAT-medium, only the immortal, HAT-resistant hybrid cells survive (Examples 3 and 9) and permanent cell lines hereafter termed DLC/tumor cell hybridomas are developed from them.

(6) The DLC/tumor cell hybridomas with therapeutic potential are then  
20 selected from all growing hybridomas. Their therapeutic potential is linked to the retention of pertinent DLC characteristics and of pertinent tumor cell characteristics. Pertinent DLC characteristics include DLC morphology, DLC surface markers, DLC genetic markers and the capacity to activate immune cells in vitro. At least one of these DLC characteristics may suffice to qualify hybridomas made of (drug-sensitive)  
25 immortal tumor cells and primary cultured DLCs, since these hybridomas necessarily inherited immortality from the tumor parent. (1°) The selection may be based on the morphologic DLC appearance of the hybridoma by scanning electron microscopy (SEM), as shown in Example 4A and Figure 1. Such an analysis can be performed on a minute sample of cells at a very early stage of hybridoma development, allowing the  
30 culture efforts to be focused on the dendritic-like hybridomas. (2°) In the absence of

morphological DLC characteristics, as in Example 10A, the expression of DLC surface markers may be used to select hybridomas with therapeutic potential. If such DLC surface markers, including namely T-cell activating molecules, are not expressed on resting hybridomas, they may nevertheless be induced by treatment with cytokines or other activating agents, as described in Example 10B. (3°) Genetic DLC markers are further used to confirm or to exclude the contribution of a T-cell, B-cell or other cell type to the hybridoma, as in Examples 4C and 10C. HLA-DR gene typing can also be used to identify blood donor genes when the tumor cell and the DLC are from distinct individuals, as in Example 10C.

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In DLC/tumor cell hybridomas involving patient's related pre-established immortal tumor cells, it is necessary to select dendritic-like hybridomas that express in addition at least one of the patient's matched tumor-associated antigens. Standard immunocytochemistry can be performed on small samples of the hybridomas to identify such tumor-associated antigens as Her2/neu for breast cancer and carcinoembryonic antigen (CEA) for colon cancer. The hybridomas identified as potentially useful are amplified in culture for complete phenotypic characterization (chromosomes, genetic markers, cell surface markers and sub-cellular morphology) and for clinical use.

20 The thirteen embodiments of our invention are briefly described as follows:

Embodiments A, B, C: primary cultured patient's tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (A), or with primary cultured DLCs differentiated from precursors derived from blood, bone marrow or other tissue (B), or with immortal DLCs (C), to yield a plurality of DLC/tumor cell hybrids that are used without selection.

Embodiments D, E: primary cultured patient's tumor cells are fused with immortal DLCs (embodiment D) or with drug-sensitive immortal DLCs (embodiment E) to yield a plurality of DLC/tumor cell hybridomas; the latter are mixed in embodiment D with unfused immortal DLC. In these embodiments, hybridomas with both DLC characteristics and tumor cell characteristics may be selected for further use.

30



Embodiments F, G: patient's immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (F), or with primary cultured DLCs differentiated from precursors (G), to yield a plurality of DLC/tumor cell hybridomas, mixed with unfused immortal tumor cells. In these embodiments, hybridomas with DLC characteristics are selected for further use.

Embodiments H, I: patient's drug-sensitive immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (H), or with primary cultured DLCs differentiated from precursors (I), to yield a plurality of DLC/tumor cell hybridomas. In these embodiments, hybridomas with DLC characteristics are selected for further use.

Embodiments J, K: Patient's related, pre-established immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (J), or with primary cultured DLCs differentiated from precursors (K), to yield a plurality of DLC/tumor cell hybridomas, mixed with unfused immortal tumor cells. In these embodiments, hybridomas with DLC characteristics and expressing in addition the patient's matched tumor-associated antigen(s) may be selected for further use.

Embodiments L, M: Patient's related, pre-established, drug-sensitive immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (L), or with primary cultured DLCs differentiated from precursors (M), to yield a plurality of DLC/tumor cell hybridomas. In these embodiments, hybridomas with DLC characteristics and expressing in addition the patient's matched tumor-associated antigen(s) may be selected for further use.

(7) The selected hybridomas are then used for inducing an anti-tumor immunity, either in vivo or in vitro, thereby contributing to the rejection of the residual tumor in the patient. For the induction of an anti-tumor immune response in vivo, the DLC/tumor cell hybridomas are irradiated or otherwise inactivated, and injected, for example sub-cutaneously, into the patient. The patient is monitored for signs of an anti-tumor immune response and for the clinical evolution of his/her cancer. In a murine model, a single injection of a living DLC/tumor cell hybridoma into syngeneic mice elicited an anti-tumor immune response as shown in Examples 5A and 5B. In

addition, multiple injections of an irradiated DLC/tumor cell hybridoma had a therapeutic effect on mice preinoculated with a lethal dose of tumor cells, as shown in Example 5C. For the induction of an anti-tumor immune response in vitro, the DLC/tumor cell hybridomas are irradiated or otherwise inactivated, and cultured with the immune cells of the patient. The activated immune cells are then re-injected into the patient. The patient is monitored for the presence of an anti-tumor immune response and for the clinical evolution of his/her cancer.

Herein, the term "dendritic-like cell (DLC)" is an operational term referring to a non-B cell present in preparations of purified or enriched dendritic cells. DLCs can be dendritic cells of myeloid origin, monocytes, cells intermediate between dendritic cells and monocytes, T-cells or other non-B cells present in the preparation.

Herein, the term "DLC/tumor cell hybrid" is defined as a fused cell which exhibits characteristics of both a DLC and the specific tumor cell of interest. Since a DLC may be a dendritic cell, a monocyte, a T-lymphocyte or another non-B cell co-purifying with dendritic cells, DLC/tumor cell hybrids may include hybrids with different phenotypic characteristics reflecting these different cell fusion partners. A plurality of DLC/tumor cell hybrids is capable of eliciting an immune response, either in vivo or in vitro, against the tumor fusion partner which makes up part of the genome of the hybrids. This capacity is not inhibited by the presence of unfused DLCs, DLC lines or unfused tumor cells or tumor cell lines.

Herein, the term "DLC/tumor cell hybridoma" is defined as an immortal hybrid cell line, which exhibits characteristics of both a DLC and the specific tumor cell of interest. Since a DLC may be a dendritic cell, a monocyte, a T-lymphocyte, and other non-B cells co-purifying with dendritic cells, DLC/tumor cell hybridomas may exhibit phenotypic characteristics of any of these cell lines. For instance, in examples below, 2 murine DLC/tumor cell hybridomas exhibited T-cell lineage characteristics, whereas 1 human DLC/tumor cell hybridoma was likely from monocytic origin. More

importantly, a DLC/tumor cell hybridoma is capable of eliciting an immune response, either in vivo or in vitro, against the tumor fusion partner which makes up part of the genome of the hybridoma.

5           Herein, the term "anti-tumor response in vivo" refers to the in vivo induction of immune effectors that confer resistance to a subsequent challenge with tumor cells, and contribute to the rejection of pre-existing tumor cells. In Example 5B, these immune effectors include cytotoxic T-lymphocytes that were detected by submitting the spleen cells of the immunized animals to an in vitro assay. In human subjects, appropriate  
10 non-invasive measures can be used for demonstrating the presence of anti-tumor immune effectors. However, the clinical course of the tumor, monitored by imaging techniques and the survival of the patient, will be the prime criterion for the evaluation of the immunotherapy.

15           Herein, the term "anti-tumor response in vitro" refers to the in vitro activation of autologous immune cells into anti-tumor immune effectors. The latter will contribute to the rejection of the pre-existing tumor cells when infused into the patient. The secretion of IL-2 by the murine T-DLC/tumor cell hybridomas (Example 6) and the secretion of GM-CSF by the human (presumed monocytic) DLC/tumor cell  
20 hybridoma may contribute to such in vitro and in vivo activation of anti-tumor immune cells.

          Herein, the term "DLC characteristics" shared by the hybridoma of the invention refers to DLC morphology, the expression of DLC surface markers, the  
25 expression of DLC genetic markers and/or the activation of immune cells.

          Herein, the term "DLC morphology" refers to a typical image observed by scanning electron microscopy. The images of the DLC/tumor cell hybridoma are compared to those of the parent tumor cell and DLC. At first glance, to one skilled in  
30 the art, it is clear that the hybridoma resembles the DLC more than the tumor cell.

Upon analysis, DLCs have irregular shapes, due to the presence of clearly-visible, flat cytoplasmic extensions like pseudopodia and veils. Hybridomas with such similar cytoplasmic extensions can be recognized as having a dendritic-like cell morphology, as illustrated in Fig. 1 (see Example 4). These data are also consistent with the possibility that other embodiments of the present invention may express these or other DLC morphological traits, since the DLC morphology of a DLC/tumor cell hybridoma is expected to mirror the particular morphology of the DLC used as a fusion partner.

Herein, the term "expression of DLC surface markers" refers to the expression of markers restricted to the DLCs used for fusion. These markers include T-cell activating molecules and other molecules. T-cell activating molecules are expressed on activated APCs; they include mainly MHC class I and class II restricting elements, as well as the family of B7 costimulatory molecules; the latter bind to the CD28/CTLA4 counter-receptor on T-cells. Other DLC surface markers include, for example, CD1a for human myeloid dendritic cells, CD14 for monocytes, and the TCR/CD3 complex for T-cells. It is shown in Example 4B (Table 1 and Figure 2) that the HY41 and HY62 hybridomas express MHC class I molecules and the TCR/CD3 complex, but neither MHC class II molecules, nor B7 costimulators. When such T-cell activating molecules are not expressed on resting hybridomas, they can sometimes be induced by exposure to cytokines or other activating agents; Example 10B illustrates such an induced expression of HLA-DR on a human DLC/tumor cell hybridoma.

Herein, the term "tumor-associated antigen" refers to a peptide derived from a protein expressed by a tumor cell which, when expressed by the hybridoma of the invention, will enable the hybridoma to elicit a tumor-specific response in vivo and/or in vitro. It also refers, by extension, to the proteins from which the antigenic peptides are derived, and to the genes encoding the antigenic proteins.

Herein, the term "activation of immune cells in vivo" refers to the immune rejection of a residual tumor, as measured by its reduction in size and by the survival of

the patient, as shown for mice in Example 5C. In vitro correlates of this in vivo state of immunity include for example the detection of blood or tissue immune cells able to kill the patient's own tumor cells in vitro. In experimental animals, the quoted expression also refers to the immune rejection of the living hybridoma, to the immune  
5 resistance to a subsequent inoculation of tumor cells, and to the presence of tumor-specific cytolytic effector cells in the lymphoid organs of the tumor-resistant animals, as shown in Example 5.

Herein, the term "activation of immune cells in vitro" refers for example to a  
10 mixed lymphocyte-tumor cell reaction, wherein the dendritic cell/tumor cell hybridoma ("the tumor cell") stimulates one of the following reactions by allogeneic T-cells ("the lymphocyte"): (1) T-cell proliferation, as measured by tritiated thymidine incorporation; (2) T-cell secretion of cytokines including for example IL-2, interferon-gamma and others, as measured by ELISA, bioassay, or reverse transcription  
15 polymerase chain reaction; (3) T-cell-mediated tumor cell lysis, as measured by chromium release assay. This term may also refer to the activation of other immune cells, like monocytes and natural killer cells, and can be measured, for example, by cytokine release or cytotoxic cell assays.

20 The following experimental examples are provided to illustrate the invention.

#### Example 1

##### Preparation of Murine Tumor-Derived Cells.

25 The P815-X2 cell line was derived from the methylcholanthrene-induced mastocytoma P815 of mouse DBA/2 origin (Dunn and Potter, 1957, J. Natl. Cancer Inst. 18: 587-601. This cell line was obtained by Thierry Boon, director of the Ludwig Institute for Cancer Research, Brussels Branch, Belgium, and recloned by his group  
30 (Uyttenhove et al, 1980, J. Exp. Med 156: 1175-1183). The subclone P1 was extensively used by T. Boon's group and given to the present inventors in 1980. A 6-thioguanine-resistant mutant was derived from P1, as described by Le et al, 1982, Proc. Natl. Acad. Sci. USA 79:7857-7861. Briefly, P1 cells were cultured in

Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Merelbeke, Belgium), in a 7% CO<sub>2</sub> atmosphere. Increasing concentrations of 6-thioguanine (Sigma, Bornem, Belgium), ranging from 1 µg/ml to 30 µg/ml were added to the culture. The final 6-thioguanine-resistant cells died in HAT-medium, i.e. in medium supplemented with 10<sup>-4</sup> M hypoxanthine, 3.8 x 10<sup>-7</sup> M aminopterin, and 1.6 x 10<sup>-5</sup> M 2-deoxythymidine (HAT supplement, Gibco BRL). Several HAT-sensitive clones were isolated by limiting dilution from these 6-thioguanine-resistant cells. A HAT-sensitive clone expressing MHC class I antigens was used in the present invention and will hereafter be called P815\*.

P815\* cells were cultured at 37°C in a 7% CO<sub>2</sub> atmosphere in tissue culture flasks (Becton Dickinson, CA) containing RPMI 1640 medium (Seromed Biochem KG, Berlin, Germany) with 10% FCS (Gibco BRL). One day before use, P815\* cells were diluted with fresh medium in order to be in exponential growth phase at the time of cell fusion.

### Example 2

#### Preparation of Murine Dendritic-Like Cells from the Spleen

The preparation of splenic DLCs was done according to a multi-step procedure initially described by Crowley et al, 1989, Cell. Immunol. 118: 108-125. This procedure was adapted as described by Sornasse et al, 1992, J. Exp. Med 175:15-21. The procedure was started one day before the fusion experiment and yielded 200,000 to 500,000 DLCs per spleen.

Briefly, DBA/2 mice were obtained from Charles River, Sulzfeld, Germany, and maintained in specific pathogen-free conditions. Animals 8 to 10 weeks old were killed by cervical dislocation; their spleens were quickly removed and kept in cold RPMI 1640 medium. The spleens were digested with collagenase (CLSIII; Worthington Biochemical Corp., Freehold, NJ) and separated into low and high density fractions on a bovine serum albumin gradient (Bovuminar Cohn fraction V

powder; Armour Pharmaceutical Co., Tarrytown, NJ). Low-density cells were cultured during 2 hours in RPMI 1640 medium with 10% FCS, and the non-adherent cells were removed by vigorous pipetting. The latter were further cultured for 1 hour in serum-free RPMI 1640 medium. The non-adherent cells were removed by gentle  
5 pipetting and cultured overnight in RPMI 1640 medium with 10% FCS. The final non-adherent fraction contained at least 95% dendritic cells, as assessed by morphology and specific staining.

### Example 3

#### 10 Fusion of Murine Tumor Cells and Dendritic-Like Cells

The procedure used to fuse HAT-sensitive tumor cells with mortal splenic DLCs was adapted from procedures used in our laboratory to generate monoclonal  
15 antibodies, as described by Franssen et al, Protides of the Biological Fluids, editor H. Peeters, Pergamon Press, Oxford, 1982, pp 645-648.

Briefly, splenic DLCs and P815\* cells were extensively washed in serum-free RPMI 1640 medium. Five million DLCs were mixed with the same number of HAT-sensitive P815\* cells in a 15 ml conical tube and centrifuged. Two hundred  $\mu$ l of a  
20 50% solution of polyethylene glycol (PEG 4000, Merck AG, Darmstadt, Germany) in RPMI 1640 medium were added dropwise to the cell pellet. The fusion was then stopped by the stepwise addition of RPMI 1640 medium.

25 The cells were washed to remove the PEG and resuspended in RPMI 1640 medium with 10% FCS. After 2 hours incubation at 37°C, the cells were centrifuged, resuspended in RPMI 1640 medium containing HAT and 10% FCS, and plated at  $10^4$  cells/well in flat-bottomed 96-well plates (Becton Dickinson, CA). The plates were seeded one day before use with a feeder layer consisting of 5,000 irradiated peritoneal  
30 cells/well. Peritoneal cells were taken from Balb/c mice and irradiated at 2,000 rads from a Cobalt 60 source before plating. The plated fusion was cultured at 37°C in a 7% CO<sub>2</sub> atmosphere. The medium (RPMI 1640 with 10% FCS and HAT) was renewed as required by cell growth. In these conditions, unfused DLCs, that are not

immortal, died within a few days of culture; unfused P815\* cells, that are immortal but HAT-sensitive, died in the HAT-containing-medium, and only hybrid cells, combining the immortality of P815\* cells with the HAT-resistance of DLCs survived and developed into growing DLC hybridomas.

5

After 3-4 weeks of culture, wells that contained a growing DLC hybridoma could be clearly identified by phase contrast microscopy. The content of a positive well was transferred into a larger well (24-well plates, Becton Dickinson, CA) previously seeded with irradiated peritoneal cells. Eventually, DLC hybridomas were transferred to small tissue culture flasks (Becton Dickinson, CA) and amplified for characterization and storage in liquid nitrogen.

10

#### Example 4:

#### 15 Selection of Murine Dendritic-Like Cell/Tumor Cell Hybridomas with Therapeutic Potential

The goal of these experiments was to select DLC/tumor cell hybridomas exhibiting at least one of the three following characteristics: (1°) a DLC morphology; (2°) DLC surface markers; (3°) DLC genetic markers.

20

##### A. Dendritic-Like-Cell Morphology

P815\* tumor cells, fresh splenic DLCs, and DLC/tumor cell hybridomas were analyzed by scanning electron microscopy (SEM). About one million cells were fixed in 2-4% glutaraldehyde for 24 hours at room temperature and washed in phosphate buffer saline. Cell suspensions were then collected on 0.2µM nylon filters, postfixed in 1% osmium tetroxide followed by 1% tannic acid mordant and uranyl acetate, with a series of saline washes in between each step. The samples were dehydrated through graded alcohols, then critical point dried from CO<sub>2</sub>. After critical point drying, the samples were mounted on aluminium stubs and sputter coated with gold using a Bio-Rad PS3 coating unit. The cells were examined at 20 kV in a Hitachi S520 scanning electron microscope.

30



Photographs of the cells are shown in Fig. 1. In these conditions, P815\* tumor cells appeared as uniform rounded cells, whose surface was spiked with numerous short microvilli (Fig. 1a). In contrast, splenic DLCs appeared as irregular cells, due to the presence of clearly-visible cytoplasmic extensions, resembling pseudopodia and veils. Furthermore, the DLC surface was not spiked with numerous microvilli, but displayed instead fewer, larger protrusions. The hybridoma cells were in general much larger than the parent P815\* cells. Many of them (like the one named HY1) looked very much like the P815\* parent, which was linked to their round regular shape and microvilli-like protrusions (Fig. 1c). In contrast, hybridomas HY41 and HY62 looked much more like the DLC parent, when considering their irregular shape and relatively bare cell surface with some large protrusions, as shown for HY41 in Fig. 1d. However, a DLC morphology may be assumed not only by dendritic cells of myeloid origin, but also by cells derived from other lineages, including cells of the B- and T- lymphocyte lineages, like follicular dendritic cells and dendritic epidermal T-cells, respectively. In order to determine the cell lineage of the DLC that fused with the P815\* tumor cell, other DLC characteristics were investigated for hybridomas HY41 and HY62.

#### B. Dendritic-Like-Cell Surface Markers

Cell surface molecules were characterized by FACS analysis, as described by Flamand et al, 1990, J. Immunol 144:2875-2882. Briefly, the cells were preincubated with 2.4G2, a rat anti-mouse Fc-receptor (Fc-R) monoclonal antibody (mAb) for 10 min prior to staining with fluorescein-coupled monoclonal antibody (fl. mAb). This preincubation was done to prevent the non-specific binding of mAb to cellular Fc-R. When unlabelled mAb were used, they were revealed by incubation with fluoresceinated anti-IgG antibodies. The labelled cells were gated for size and side scatter to eliminate dead cells and debris, and analyzed on a Facscan (Becton Dickinson, CA).

The results are summarized in Table 1. No T-cell activating molecules or other dendritic-cell-associated molecules were expressed by the HY41 and HY62 hybridomas. However, a fraction of the cells of both hybridomas expressed surface CD3 $\epsilon$  chains of the T-cell receptor (TCR), suggesting that they were T-lymphocyte/tumor cell hybridomas. After cloning by limiting dilution, CD3 $^{+}$  and CD3 $^{-}$  subclones were isolated from both hybridomas. Figure 2 shows that the HY41 and HY62 CD3 $\epsilon^{+}$  subclones were also labeled by a fl mAb specific for the V  $\beta$ 8 domain of the TCR, whereas P815\* tumor cells and the CD3 $\epsilon$ -subclones remained unstained. These results showed that the HY41 and HY62 hybridomas expressed an  $\alpha/\beta$  TCR, and hence had incorporated a dendritic-like T-lymphocyte. However, neither CD4 or CD8 were expressed by the hybridomas. In order to confirm these cell surface marker studies, genetic marker studies were undertaken.

Table 1. Cell Surface Markers of Murine Dendritic-Like-Cell/Tumor Cell Hybridomas  
HY41, HY62 and Parent Cells

Reagents	Surface markers	DLCs <sup>1</sup>	P815*	HY41	HY62
<u>Present on DLCs and P815*</u>					
31.3.4 mAb	MHC class I Kd	+	+	+	+
34.4.20 mAb	MHC class I Dd	+	+	+	+
30.5.7 mAb	MHC class I Ld	+	+	+	+
3E2 fl mAb	ICAM-1 (CD54)	+	+	-	-
<u>Present on P815* only</u>					
2.4G2 mAb	Fc-R	-	+	-	-
<u>Present on DLCs only</u>					
T-cell activating molecules:					
14.4.4 fl.mAb	MHC class II	+	-	-	-
16-10A1 fl mAb	B7-1 (CD80)	+	-	-	-
GL1 fl mAb	B7-2 (CD86)	+	-	-	-
CTLA4- human Ig	CTLA4-ligand	+	-	-	-
M1/69 fl mAb	HSA (CD24)	+	-	-	-
Other molecules:					
N418 fl mAb	N418 (CD11c)	+	-	-	-
145-2 C11	CD3e	nd <sup>2</sup>	-	+	+
F23-1	TCR V $\beta$ 8 chain	nd	-	+	+
H129.19	CD4	nd	-	-	-
53-6.7	CD8a	nd	-	-	-

- 5 <sup>1</sup>: By cell scatter and cell surface marker analyses, DLCs contained more than 95% dendritic cells;  
<sup>2</sup>: nd: not detectable  
31.3.4, 34.4.20, 30.5.7: mouse anti-mouse H2-K<sup>d</sup>, D<sup>d</sup> and L<sup>d</sup> mAb, respectively; Ozato et al, 1980, J. Immunol. 124:533-;  
3E2: hamster anti-ICAM-1, from Pharmingen, San Diego, CA  
10 2.4G2 : rat anti-mouse Fc-gamma-RII/III mAb (Unkeless, 1979, J. Exp Med 150:580-586;  
14.4.44: mouse anti-I-E<sup>d</sup> fluorescein-coupled mAb (fl mAb); Ozato et al, 1980, J. Immunol. 124:533-  
16-10A1: rat anti-B7-1 fl mAb; Razi-Wolf et al, 1993, Proc. Natl. Acad. Sci. USA 90:11182-11186;  
GL1: hamster antiB7-2 fl mAb; Hathcock et al, 1993, Science 262:905-907;  
CTLA4-human IgG fusion protein: Linsey et al, 1991, J. Exp. Med 174:561-569;  
15 M1/69: Rat anti-HSA, from Pharmingen, San Diego, CA.  
N418: hamster anti-mouse CD11c; Metlay et al, 1990, J. Exp. Med 171:1753-1771;  
145-2C11: hamster anti-mouse DC3e fl mAb; Leo et al, 1987, Proc.Natl.Acad.Sci.USA 84:1374  
F23.1: mouse anti-mouse TCR V  $\beta$ 8 fl mAb from ATCC, Bethesda MD.  
H129.19: rat anti-mouse CD4 fl mAb, from Gibco BRL, Gaithersburg, MD.  
20 53-6.7: rat anti-mouse CD8a fl mAb, from Gibco BRL, Gaithersburg, MD.  
ND: not detectable

### C. DLC Genetic Markers

First, Southern blot analysis was used to analyse the rearrangement status of the TCR genes in genomic DNA from the HY41 hybridoma. The mouse T-cell hybridoma 13.26.8-H6 was used as a reference for rearranged TCR genes (Ruberti et al, 1992, J. Exp. Med. 175: 157-162), and P815\* mastocytoma cells as well as DBA/2 spleen cells were taken as controls for germ line TCR genes. Genomic DNA was extracted from  $2 \times 10^7$  cultured cells and from spleens, using the Genome DNA Kit (Bio 101, CA, USA) according to the manufacturer's instructions. Ten  $\mu\text{g}$  of DNA were digested for  $\pm 4$  hours with various restriction enzymes, separated on a 1% agarose gel and transferred to a nylon membrane (Qiabran Nylon plus, Qiagen, Hilden, Germany) according to standard procedures. The blot was hybridized to a DIG-labeled synthetic oligonucleotide of 50 bases targeted to the first exon of the constant region of the mouse TCR  $\beta$  chain and processed for chemiluminescent detection using Boehringer Mannheim's DIG detection kit. The results showed that the HY41 genome contained a rearranged TCR  $\beta$  chain gene, which is a hallmark of T-cell lineage commitment (not shown).

Next, the Polymerase Chain Reaction (PCR) was used to detect rearranged V  $\beta$ 8-C $\beta$  sequences of the TCR in genomic DNA. The upstream primer was targeted to bases 47-66 with respect to the ATG initiation codon of the mouse V  $\beta$ 8 region (5'-AACACATGGAGGCTGCAGTC-3') and the downstream primer was targeted to bases 141-160 of the first exon of the C $\beta$  region (5'-GTGGACCTCCTTGCCATTCA-3'). The PCR was carried out essentially according to the instructions of Boehringer Mannheim's Long Range Expand PCR System. Analysis of the PCR products on a 1% agarose gel stained with ethidium bromide is shown in Figure 3. A fragment with the expected length (4.5 to 5 kb) of the rearranged V  $\beta$ 8-C $\beta$  fragment is clearly seen in DNA from the T-cell hybridoma 13-26-8-H6 (lane T), used as a positive control, as well as in DNA from the HY41 and HY62 hybridomas (lanes 41 and 62); this fragment is not amplified in DNA from P815\* tumor cells and from spleen cells (lanes P and S), used as negative controls. These results confirm that the

DLC that fused with a P815\* tumor cell to yield the HY41 and HY62 hybridomas was a T-lymphocyte expressing an  $\alpha/\beta$  TCR receptor, including the V $\beta$ 8 domain. These hybridomas will hereafter be termed T-DLC/tumor cell hybridomas.

5 In conclusion, the HY41 and HY62 T-DLC/tumor cell hybridomas were selected for further studies because of their DLC morphology and T-lymphocyte lineage. In both hybridomas, the T-lymphocyte fusion partner was a rare and undetectable contaminant of the splenic DLC preparation. In view of the complex genetic regulations controlling CD4 and CD8 expression in somatic cell hybrids  
10 (Wilkinson et al, 1991, J. Exp. Med. 174: 269-280), it is impossible to determine a posteriori if the fusing T-cell was a CD4+, CD8+, or CD4-CD8- "double negative" T-cell. However, whatever the sublineage of T-lymphocyte involved, the next step was to determine the in vivo immunogenicity of these T-DLC/tumor cell hybridomas.

#### 15 Example 5

##### In vivo Immunogenicity of Murine T-Dendritic-Like-Cell/Tumor Cell Hybridomas

The goal of these experiments was to determine if the hybridomas induced an efficient  
20 immune rejection in vivo, as measured by the following criteria: (1°) rejection of the hybridomas by immunocompetent mice; (2°) vaccination with the hybridomas against a subsequent inoculation of tumor cells; (3°) treatment with the hybridomas after prior inoculation of tumor cells.

#### 25 A. Immune Rejection of T-DLC/Tumor Cell Hybridomas

Groups of 10 to 12 DBA/2 mice were injected intra-peritoneally with 500,000 living cells of the P815\* tumor or of the HY41 hybridoma. Injected animals included mice immunosuppressed by sub-lethal irradiation as well as immunocompetent mice. All irradiated animals died from their tumor within four weeks of inoculation, showing  
30 that the HY41 and P815\* cell lines were very similar in their tumorigenicity (Fig. 4). In contrast, 9/12 (75%) immunocompetent animals injected with the HY41 hybridoma survived two months after inoculation, when only 2/10 (20%) mice had survived the

parental tumor injection. This experiment showed that the HY41 hybridoma was as tumorigenic as the parent tumor in irradiated mice, but more immunogenic than P815\* in immunocompetent mice. Similar results were obtained with hybridoma HY62 (not shown).

5

#### B. Induction of Tumor Resistance by Murine T-DLC /Tumor Cell Hybridomas

The 9 surviving HY41-treated mice, as well as 9 untreated animals, were challenged intra-peritoneally with 500,000 P815\* cells. All (9/9) untreated mice died from their tumor within six weeks of inoculation, showing that the tumor cell injection was lethal for unimmunized animals. By contrast, 7/9 HY41-treated animals were still alive 6 weeks after tumor challenge, and 4/9 of them survived for at least three months (Fig. 5). These results strongly suggested that prior treatment of syngeneic mice with living HY41 DC hybridoma cells induced a memory immune response against the parent P815\* cell line, conferring tumor resistance to 44% of the treated animals. A similar tumor-resistance could be induced by the injection of living HY62 hybridoma cells (not shown).

The spleens of the 4 P815\*-resistant mice were tested in vitro for the presence of anti-P815\* cytotoxic T-cells, as described by Moser et al, 1987, J. Immunol 138: 1355-1362. Briefly, spleen cell suspensions were stimulated in vitro during 5 days with the irradiated P815\* cells, in order to induce a measurable memory response. They were then used as effector cells on chromium-loaded P815\* and L1210 target cells. The latter have the same MHC class I haplotype (H-2d) as P815 cells. At several effector/target ratios, the spleen cells of the untreated animals completely failed to lyse the P815\* and the L1210 target cells (Figs 6A and 6B). In contrast, the spleen cells from the 4 P815\*-resistant mice lysed efficiently and specifically the P815\* targets, without showing any significant activity on the L1210 targets. These results showed that the HY41-treated, P815\*-resistant animals were able to mount a strong and tumor-specific cytolytic response upon in vitro restimulation.

30

### C. Induction of Tumor Treatment by Murine T-DLC/Tumor Cell Hybridomas.

In this experiment, 40 DBA/2 mice received an ip injection of  $2 \times 10^5$  P815\* tumor cells. Seven days later, the mice were divided into 4 groups of 10 animals; the first group was left untreated while the 3 other groups were treated by 4 weekly ip injections of  $2 \times 10^6$  irradiated (15,000 F) P815\*, HY41 or HY62 cells. The data are presented in figure 7. Untreated animals all died within 7 weeks of tumor inoculation, and 20% of the mice treated with irradiated P815\* tumor cells survived, confirming the weak immunogenicity of the P815 tumor cell line. In contrast, 60% and 40% of the mice treated with irradiated HY41 and HY62 hybridoma cells, respectively, survived the prior injection of a lethal dose of P815\* cells. These data showed that hybridoma HY41, and to a lesser extent hybridoma HY62, could induce the immune rejection of an established tumor. However, the mechanism leading to such an efficient in vivo immune rejection remained unclear. One possibility that was explored concerned the secretion of immunomodulating cytokines.

#### Example 6:

##### In vitro analysis of cytokine expression by T-DLC/tumor cell hybridomas

The goal of these experiments was to determine whether the HY41 and HY62 hybridomas synthesized some cytokines that could account, at least in part, for their in vivo immunogenicity. Total RNA was prepared from activated spleen cells, from P815\* tumor cells and from the HY41 and HY62 hybridomas according to standard procedures. The Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and cytokine-specific primers were used to amplify IL-2, IL-4, IL-10 and interferon  $\gamma$  (IFN $\gamma$ ) mRNA sequences, as described by De Wit et al, J. Immunology, 1993, 150: 361-366. The primers used to amplify IL-12 p40 sequences were 5'-TTCAACATCAAGAGCAG TAGC-3' and 5'-GGAGAAGTAGGAATGGGGAGT-3'. Analysis of the RT-PCR products on ethidium bromide-stained agarose gels showed that P815\* tumor cells constitutively expressed IL-4 mRNA and that the HY41 and HY62 hybridomas constitutively expressed IL-2 and IL-4 mRNAs, but not

IL-10, IL-12, and IFN $\gamma$  mRNAs. These cytokine mRNAs were nevertheless detected in activated spleen cells, used as a positive control. In conclusion, these data showed that the HY41 and HY62 T-DLC/tumor cell hybridomas constitutively expressed IL-4 like the parent P815\* tumor cell, and IL-2, like the parent T-lymphocyte. These cytokines, if secreted in vivo, may at least partially contribute to the immunogenicity of the hybridomas.

#### Example 7

##### Preparation of Human Tumor-Derived Cells

10           The human 143B thymidine kinase negative osteosarcoma cell line (hereafter termed 143B) is a HAT-sensitive cell line that was purchased from the ATCC (CRL n° 8303). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2% penicillin/streptomycin, 1% sodium pyruvate (all from Gibco BRL, Merelbeke, Belgium) and 0.015 mg/ml of 5-bromo-2'-deoxyuridine (Sigma Chemical  
15   Co, St Louis, MO). One day before fusion, the cells were diluted with fresh medium in order to be in exponential growth phase.

#### Example 8

##### Preparation of human Dendritic-Like Cells from Peripheral Blood.

20           Dendritic cells were differentiated in vitro from adherent blood precursors, using an adaptation of the technique described by Romani et al, 1994, J. Exp .Med. 180: 83-93. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat of a healthy donor by density gradient centrifugation on lymphoprep (Gibco BRL). Adherent cells were prepared by plating 10<sup>7</sup> PBMC on 6-well tissue  
25   culture plates in 3 ml RPMI supplemented with 200 mM L-Glutamine, 50  $\mu$ M Mercaptoethanol and 10% FCS. After 2 hours incubation at 37°C, the non-adherent cells were discarded by a very gentle rinse, and the adherent cells were further cultured in the above-described medium supplemented with GM-CSF (Leucomax, 800 U/ml) and IL-4 (Genzyme, 500 U/ml), at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.  
30   After 7 days of culture, DLCs were recovered and characterized by cell scatter and cell



surface marker analysis. The DLCs used for fusion contained 50% of monocytic-like cells, expressing CD14 but not CD1a or CD1c, as well as 38% of T lymphocytes, 4% of NK cells and 8% of B lymphocytes.

5

### Example 9

#### Fusion of Human Tumor Cells and Dendritic-Like Cells

The procedure used to fuse HAT-sensitive tumor cells with DLCs was adapted from procedures used to generate monoclonal antibodies (Current Protocols in Immunology, chapter 2.5.4). The 143B tumor cells and the DLCs were extensively washed in serum-free medium (RPMI 1640);  $2 \times 10^6$  DLCs were mixed with  $1 \times 10^6$  143B osteosarcoma cells and centrifuged. The pellet was resuspended in 500  $\mu$ l of a 50% solution of polyethylene glycol (PEG 4000, Gibco) in Dulbecco's phosphate buffered saline without  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  (ref. 14030035). After 1 minute, the PEG was progressively diluted by the slow and progressive addition of serum-free medium. The cells were washed free of PEG and resuspended in RPMI 1640 with 10% FCS. They were eventually plated at  $2 \times 10^4$  cells/well in flat-bottomed 96-well plates (Falcon, Becton Dickinson) and cultured in a 5%  $\text{CO}_2$  atmosphere at 37°C. HAT medium was added to the wells 24 hours after fusion and renewed every two days. In these conditions, unfused DLCs died within 2-3 weeks of culture, unfused 143B osteosarcoma cells died in HAT-medium and only hybrid cells combining the immortality of the tumor cell with the HAT-resistance of a DLC survived and developed into growing cell lines. After 3-4 weeks of culture, wells containing growing cell lines were clearly identified by phase contrast microscopy. Their contents were transferred into larger wells and eventually into culture flasks for amplification. Culture stocks were frozen in liquid nitrogen before analysis.

20  
25  
30

### Example 10

Identification of Human Dendritic-Like Cells/Tumor Cell Hybridomas with Therapeutic Potential

The goal of these experiments was to identify human DLC/tumor cell hybridomas presenting at least one of the three following characteristics: (A) DLC morphology; (B) DLC surface markers; (C) DLC genetic markers.

5 A. DLC morphology

The 143B osteosarcoma cells and a series of hybridoma cells were analyzed by SEM, as described in example 4. Comparison of the parent cells and hybridoma cells showed that none of the hybridomas analysed, including F3BG10 cells, displayed morphologic dendritic-like features. In the absence of such features, other dendritic-like features were analyzed, namely the presence of DLC surface markers.

B. DLC surface markers

Cell surface markers were analysed as described in Example 4. Results are summarized in Table 2. None of the tested hybridomas, including F3BG10 cells, expressed the T-cell activating molecules HLA-DR, B7.1, and B7.2. However, they expressed HLA class I, ICAM-1 (CD54) and LFA-3 (CD58), which were also present on the 143B tumor cells. They failed to express typical dendritic-cell markers like CD1a and CD1c, as well as markers specific for T-cells (CD3), B-cells (CD19), NK cells (CD56) and monocytes (CD14).

20

Since the hybridomas tested failed to express constitutively T-cell activating molecules, they were stimulated with a variety of cytokines in order to induce such expression. It was found that 40% of the F3BG10 hybridoma cells were induced to express varying amounts of surface HLA-DR after a 24 hour incubation with interferon  $\gamma$ . After cloning by limiting dilution, subclones were tested for their capacity to express induced HLA-DR. Figure 8 shows that at least 90% of H12 cells clearly expressed induced HLA-DR, which greatly increases their immunogenic potential.

25

Table 2. Cell Surface Markers of Human Dendritic-Like-Cell/Tumor Cell Hybridoma  
F3BG10 and Parent Cells

Reagents from	Surface markers	DLCs <sup>1</sup>	143B	F3BG10
<u>Present on DLCs and 143B</u>				
Pharmingen	HLA class I	+	+	+
Immunotech	ICAM-1 (CD54)	+	+	+
Becton Dickinson	LFA-3 (CD58)	+	+	+
<u>Present on DLCs only</u>				
T-cell activating molecules:				
Becton Dickinson	HLA-DR	+	-	- <sup>2</sup>
Innogenetics	B7.1 (CD80)	+	-	-
Pharmingen	B7.2 (CD86)	+	-	-
Other molecules:				
Immunotech	CD1a	+	-	-
Immunotech	CD1c	+	-	-
Becton Dickinson	CD14	+	-	-
Becton Dickinson	CD2	+	-	-
Becton Dickinson	CD3	+	-	-
Becton Dickinson	CD19	+	-	-

5

<sup>1</sup>: By cell scatter and cell surface marker analyses, DLCs contained 50% monocytes, 38.% T-lymphocytes and 4% NK cells; the suspension also contained 8% of B-lymphocytes.

<sup>2</sup>: HLA-DR expression could be induced in 40% of F3BG10 cells and in 90% of its H12 subclone by incubation with interferon  $\gamma$ .

10

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### C. DLC genetic markers

The goal of this first experiment was to determine whether the F3BG10 hybridoma had been generated by the fusion of a DLC with the 143B tumor cell, and to exclude that it was a revertant 143B tumor cell clone, that had become resistant to HAT-medium by mutation. This was done by typing the HLA-DR genes of the blood donor, of the 143B tumor cell and of the F3BG10 hybridoma. Genomic DNA was prepared according to standard procedures from 143B tumor cells, from the PBMC of the blood donor and from F3BG10 hybridoma cells. These DNAs were submitted to a non-isotypic HLA-DR B oligotyping method, described for the typing of DR B 1, 3, 4, 5 alleles by Buyse et al. 1993, Tissue Antigens 41: 1-4. The polymorphic second exon of the corresponding genes was amplified by PCR, and biotinylated nucleotides were incorporated into the amplifying fragments during this procedure. The PCR products were hybridized with a combination of 31 sequence-specific oligonucleotide probes, immobilized in parallel lines on membrane strips. After a stringent wash, streptavidin-labelled alkaline phosphatase was added to mark the biotinylated DNA fragments. The addition of the BCIP/NBT chromogen resulted in a colored precipitate. All reagents were part of the Innolipa DRB Key kit purchased from Innogenetics (Zwijndrecht, Belgium). The F3BG10 lane showed a mixture of bands corresponding to alleles present in the 143B osteosarcoma cells and in the PBMC of the blood donor, confirming that F3BG10 hybridoma was a DLC/tumor cell hybridoma.

The goal of the second experiment was to investigate whether it was a T-lymphocyte or a B-lymphocyte that fused with a 143B tumor cell to yield the F3BG10 hybridoma. Genomic DNA was tested for the presence of rearranged T-Cell Receptor (TCR) genes or B-cell Receptor (BCR) genes by Southern blot analysis with TCR-specific or BCR-specific probes. Standard procedures were used. Briefly, samples of 10 µg of DNA were submitted to overnight digestion at 37°C with different restriction enzymes. Hind3, Xba1 and Hind3 + Xba1 were used for the TCR rearrangements and EcoR1, Hind3 and Hind3 + BamH1 were used for BCR rearrangements. The restriction fragments were separated by electrophoresis on a 1% agarose gel,

transferred to nitrocellulose, baked and hybridized with probes specific for either the  $\beta$  chain gene of the TCR, or for a segment of the J gene of the Ig heavy chain of B lymphocytes. The results clearly showed that there were only germ line TCR genes and germ line BCR genes in the genomic DNA of the F3BG10 hybridoma. These data  
5 excluded that the DLC/tumor cell hybridoma F3BG10 was produced by the fusion of the tumor cell with a T-lymphocyte or a B-lymphocyte. The DLC fusion partner could have been a monocyte, a dendritic cell, an intermediate cell between these two cells, a natural killer cell or another unidentified non-B cell. Because the pattern of cytokine secretion could provide indications on the cell lineage of the fusion partner, we  
10 investigated cytokine secretion by F3BG10 cells.

### Example 11

#### In vitro analysis of cytokine secretion by human DLC/tumor cell hybridoma

15

The culture supernatants of the F3BG10 hybridoma cells and of the 143B tumor cells were assayed by ELISA for the presence of various cytokines, before and after 36 hours of culture in the presence of various stimuli including interferon  $\gamma$ , TNF $\alpha$ , GM-CSF and combinations of these. The results showed that the 143B  
20 osteosarcoma cells and the F3BG10 hybridoma cells secreted similar levels of IL-6 and IL-8, that could be increased for both cytokines by stimulation with the above-mentioned cytokines. In addition, the F3BG10 cells but not the tumor cells secreted significant levels of GM-CSF, that could be increased by stimulation. Neither the tumor cells or the hybridoma cells secreted detectable levels of IL-1 $\beta$ , IL-10, IL-12  
25 and TNF $\alpha$ . These results showed that the F3BG10 hybridoma secreted IL-6 and IL-8 like the parent tumor cell, and GM-CSF like the parent DLC. Since it was excluded that the latter was a T-lymphocyte, this result suggested that the fusion partner was a monocyte.

What is claimed is:

1. A plurality of dendritic-like cell/tumor cell hybrids which is capable of inducing an anti-tumor response.
- 5 2. The plurality of hybrids of claim 1 which is capable of activating immune cells in vivo against a specific tumor.
3. The plurality of hybrids of claim 1 which is capable of activating immune cells  
10 in vitro against a specific tumor.
4. The plurality of hybrids of any one of claims 1-3 which is human in origin.
5. A dendritic-like cell/tumor cell hybridoma which is capable of inducing an anti-  
15 tumor response.
6. The hybridoma of claim 5 which is capable of activating immune cells in vivo against a specific tumor.
- 20 7. The hybridoma of claim 5 which is capable of activating immune cells in vitro against a specific tumor.
8. The hybridoma of any one of claims 5-7 which is human in origin.
- 25 9. A method for producing a plurality of dendritic-like cell/tumor cell hybrids useful for the induction of an anti-tumor response, said method comprising;
  - (a) providing a sample of a tumor against which said response is needed,
  - (b) preparing a primary cell culture comprising tumor cells derived from said tumor sample,
  - 30 (c) providing autologous or HLA-compatible allogeneic dendritic-like cells,

(d) fusing said dendritic-like cells with said tumor cells to produce a plurality of hybrids.

10. The method of claim 9 wherein the dendritic-like cells of step (c) are produced  
5 by culturing precursors in the presence of cytokines.

11. The method of claim 9 wherein the dendritic-like cells of step (c) are members of an immortal cell line.

10 12. A method for producing a dendritic-like cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising;

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary culture of said tumor sample to provide tumor cells,

15 (c) deriving an immortal cell line from said tumor cells to produce immortal tumor cells,

(d) providing autologous or HLA-compatible allogeneic dendritic-like cells,

(e) fusing said dendritic-like cells with said immortal tumor cells to produce  
20 a plurality of hybridomas,

(f) selecting from said plurality of hybridomas a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic-like cell morphology, dendritic-like cell surface markers, dendritic-like cell genetic markers and immune cell activation in vitro.

25

13. The method of claim 12 wherein the dendritic-like cells of step (d) are produced by culturing precursors in the presence of cytokines.

14. The method of claim 12 or claim 13 wherein the immortal tumor cells of step (c) are drug-sensitive, said method further comprising, after step (e), killing unfused drug-sensitive immortal tumor cells by exposure to said drug.

5 15. A method for producing a dendritic-like cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising;

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary cell culture comprising tumor cells derived from said tumor sample,

10 (c) providing an immortal cell line comprising immortal autologous or HLA-compatible allogeneic dendritic-like cells,

(d) fusing said immortal dendritic-like cells with said tumor cells to produce a plurality of hybridomas, and

(e) selecting from said plurality of hybridomas a hybridoma which exhibits  
15 at least one characteristic selected from the group consisting of tumor cell morphology, tumor cell surface markers, tumor cell chromosomal and genetic markers.

16. The method of claim 15 further comprising selecting from said plurality of hybridomas a hybridoma which exhibits at least one characteristic selected from the  
20 group consisting of dendritic-like cell morphology, dendritic-like cell surface markers, dendritic-like cell genetic markers and immune cell activation in vitro.

17. The method of claim 15 or 16 wherein the dendritic-like cells of step (c) are drug sensitive, said method further comprising, after step (d), killing unfused drug-  
25 sensitive immortal dendritic-like cells by exposure to said drug.

18. A method for producing a dendritic-like cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising;

(a) providing a sample of a tumor against which said response is needed,

30 (b) analyzing tumor-associated antigens of said tumor sample,



- (c) providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample,
- (d) providing autologous or HLA-compatible allogeneic dendritic-like cells,
- (e) fusing said dendritic-like cells with said immortal tumor cells to produce
- 5 a plurality of hybridomas, and
- (f) selecting from said plurality of hybridomas a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic-like cell morphology, dendritic-like cell surface markers, dendritic-like cell genetic markers and immune cell activation in vitro.

10

19. The method of claim 18 further comprising selecting from said plurality of hybridomas a hybridoma which expresses at least one tumor-associated antigen in common between the immortal tumor cells and the tumor against which an immune response is needed.

15

20. The method of claim 18 or 19 wherein the dendritic-like cells of step (d) are produced by culturing precursors in the presence of cytokines.

20

21. The method of any one of claims 18-20 wherein the tumor cells of step (c) are drug-sensitive, said method further comprising, after step (e), killing unfused drug-sensitive immortal tumor cells by exposure to said drug.

25

22. A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject a plurality of dendritic-like cell/tumor cell hybrids.

30

23. A method for producing an anti-tumor response in vitro, said method comprising co-cultivating in vitro a plurality of dendritic-like cell/tumor cell hybrids of claim 3 with immune cells from a mammalian subject in need of anti-tumor treatment.

24. A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject autologous immune cells activated in vitro by the method of claim 23.
- 5 25. A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject a dendritic-like cell/tumor cell hybridoma.
26. A method for producing an anti-tumor response in vitro, said method  
10 comprising co-cultivating in vitro a dendritic-like cell/tumor cell hybridoma of claim 7 with immune cells from a mammalian subject in need of anti-tumor treatment.
27. A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject  
15 autologous immune cells activated in vitro by the method of claim 26.



FIG. 1b

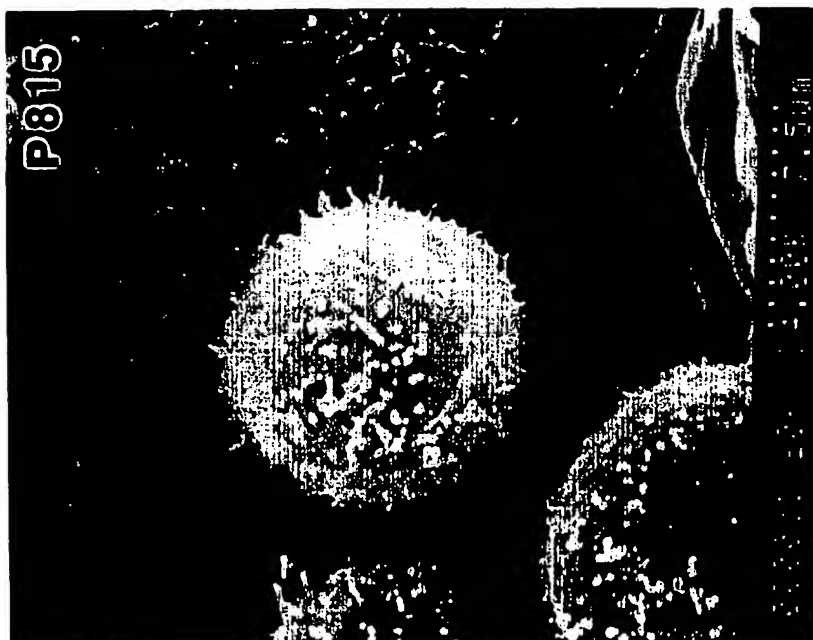


FIG. 1a

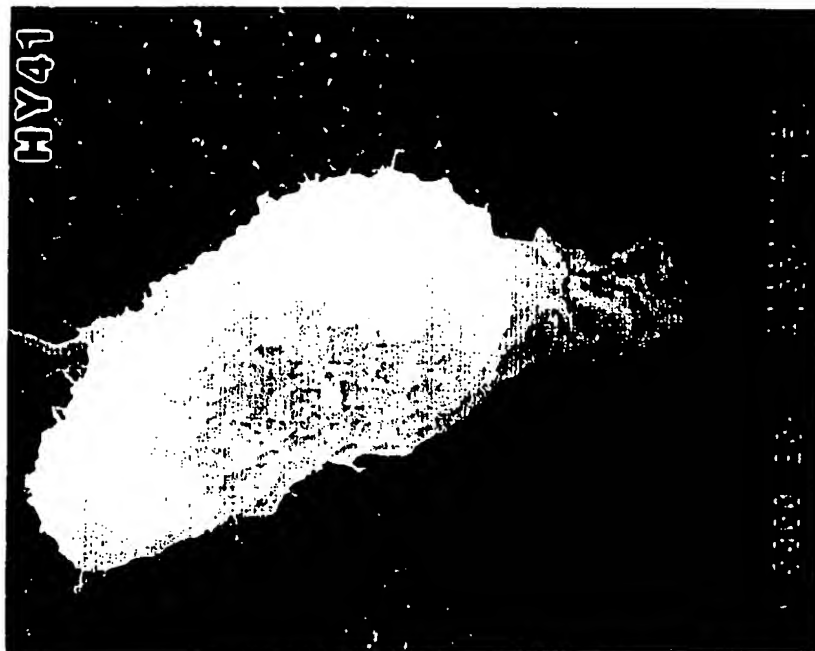


FIG. 1d

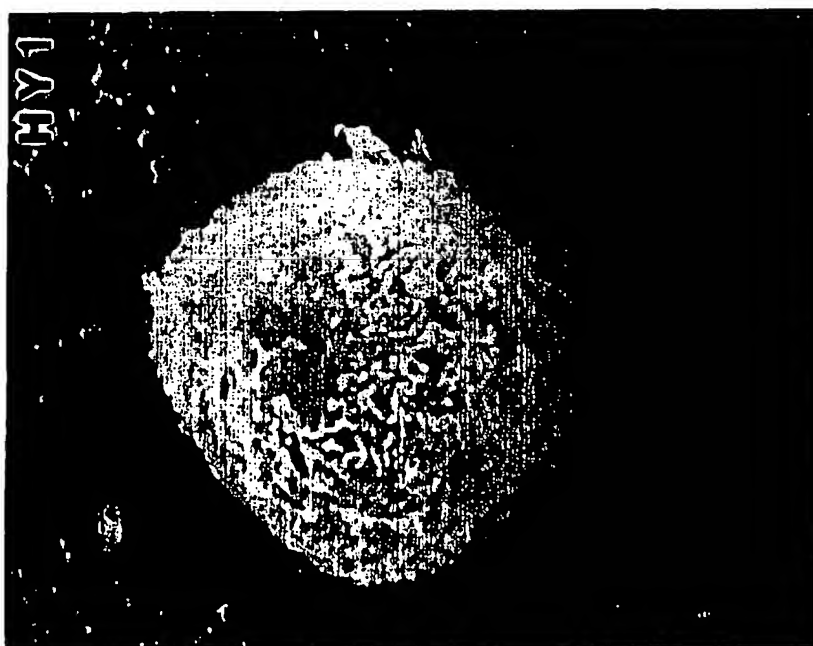


FIG. 1c

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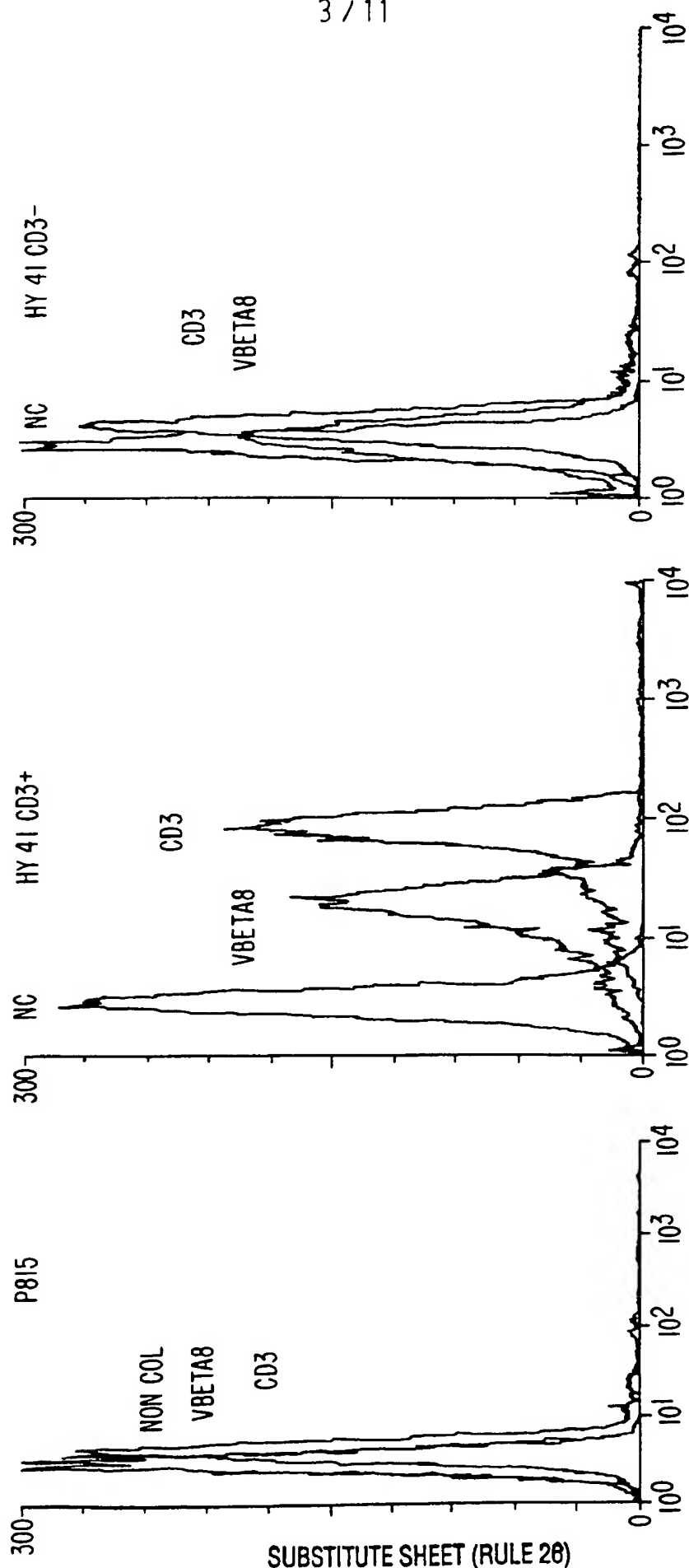


FIG. 2a

FIG. 2b

FIG. 2c

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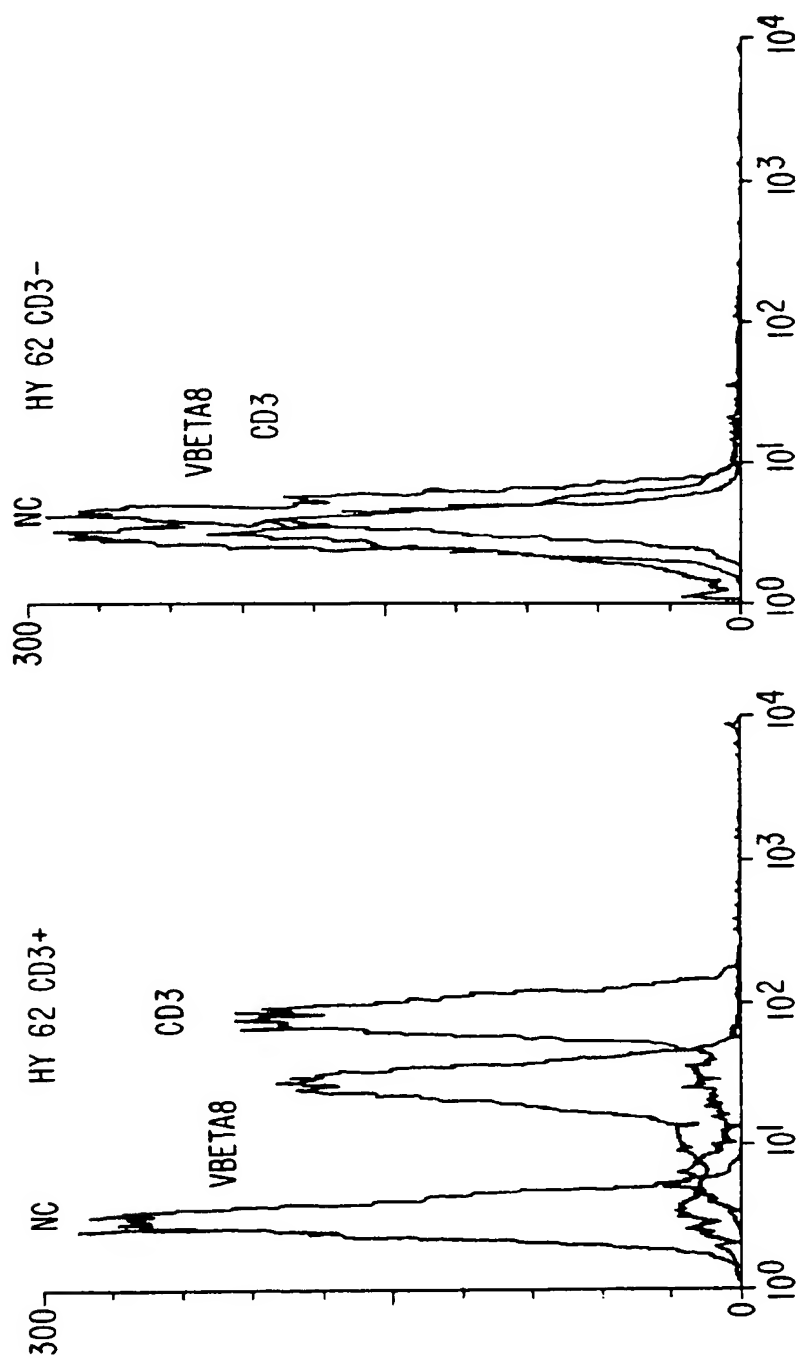


FIG. 2e

FIG. 2d

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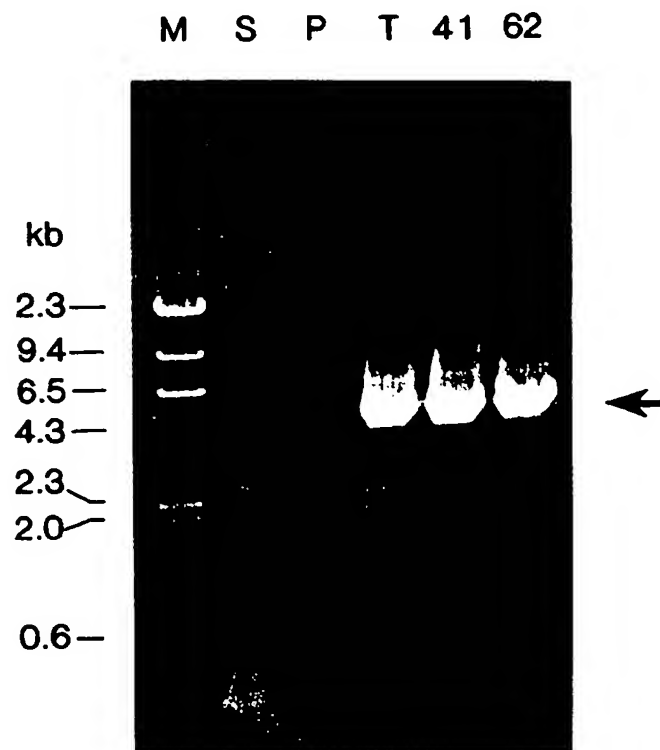


FIG. 3

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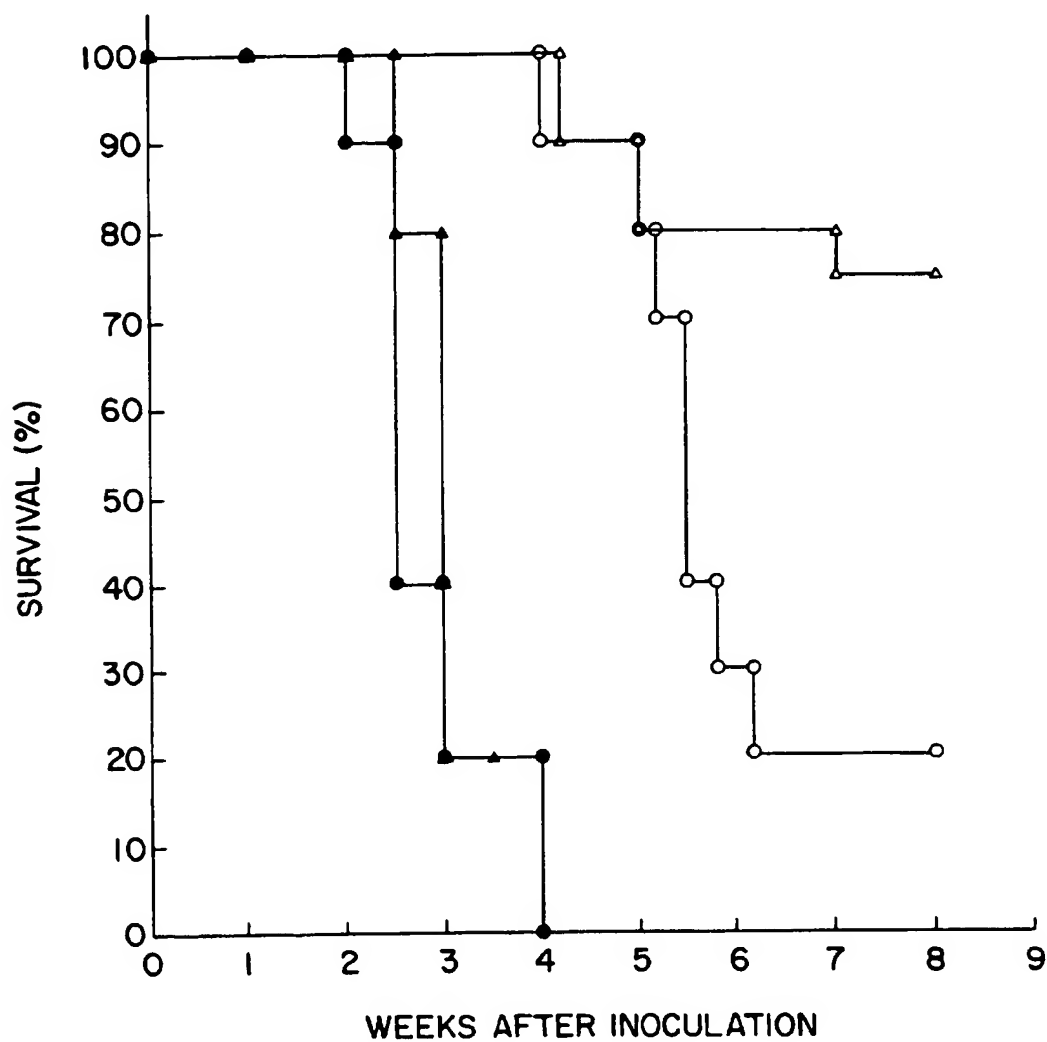


FIG. 4



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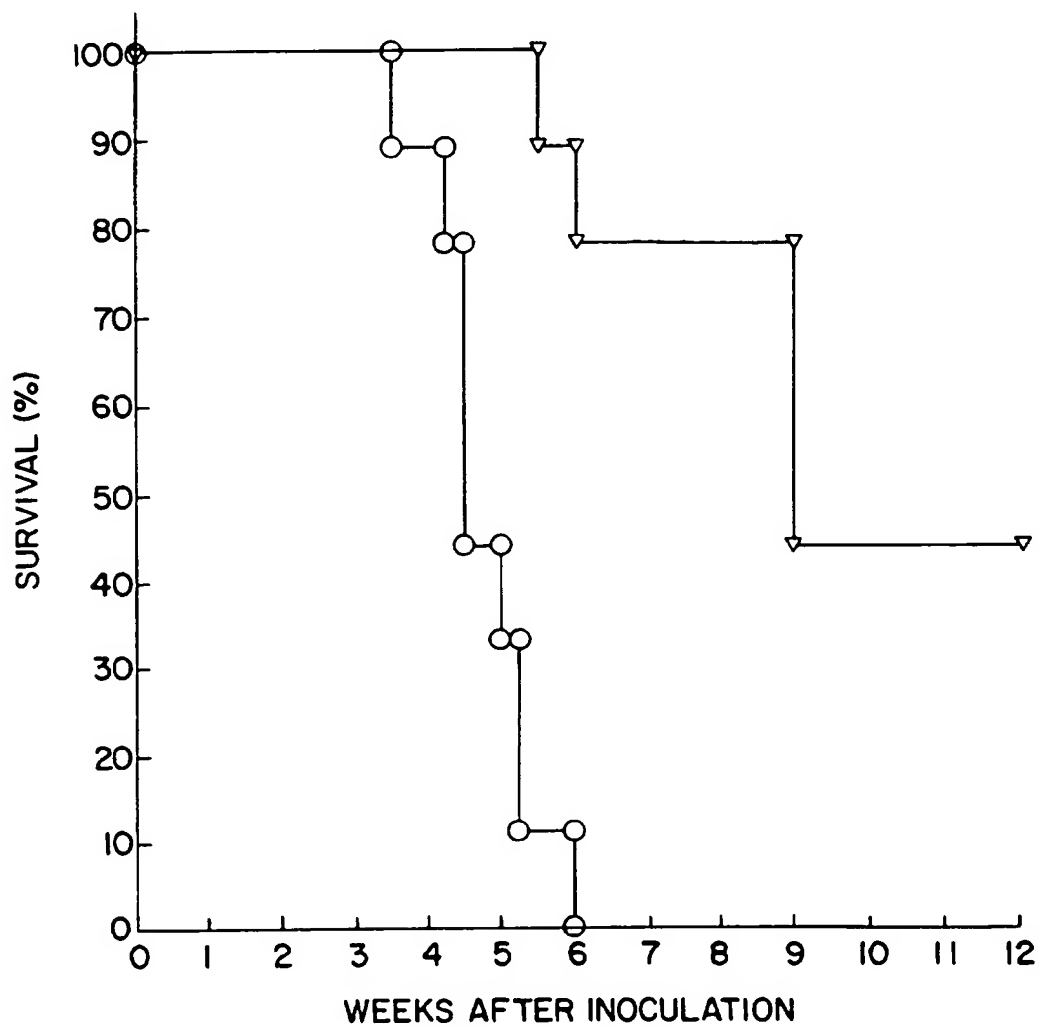
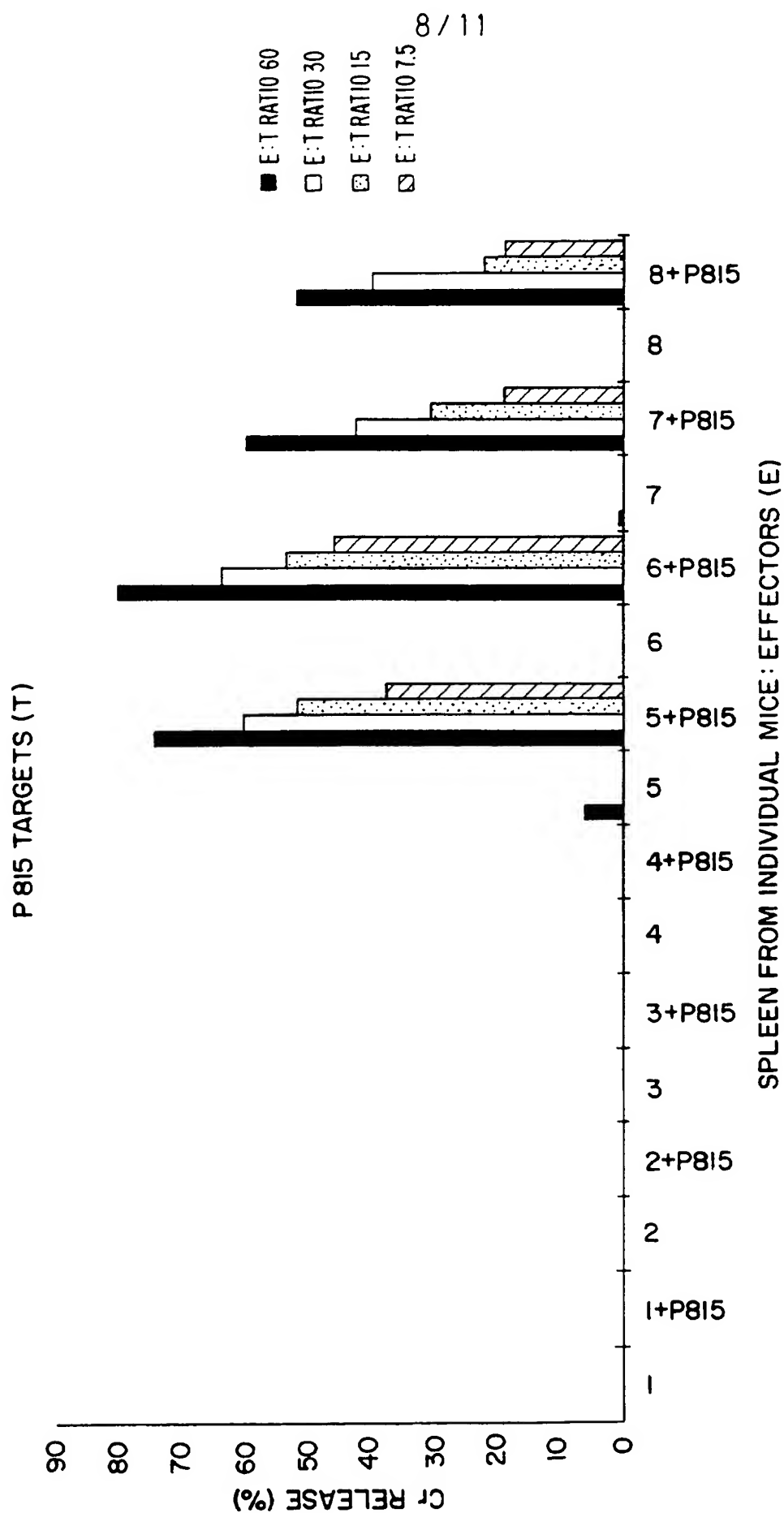
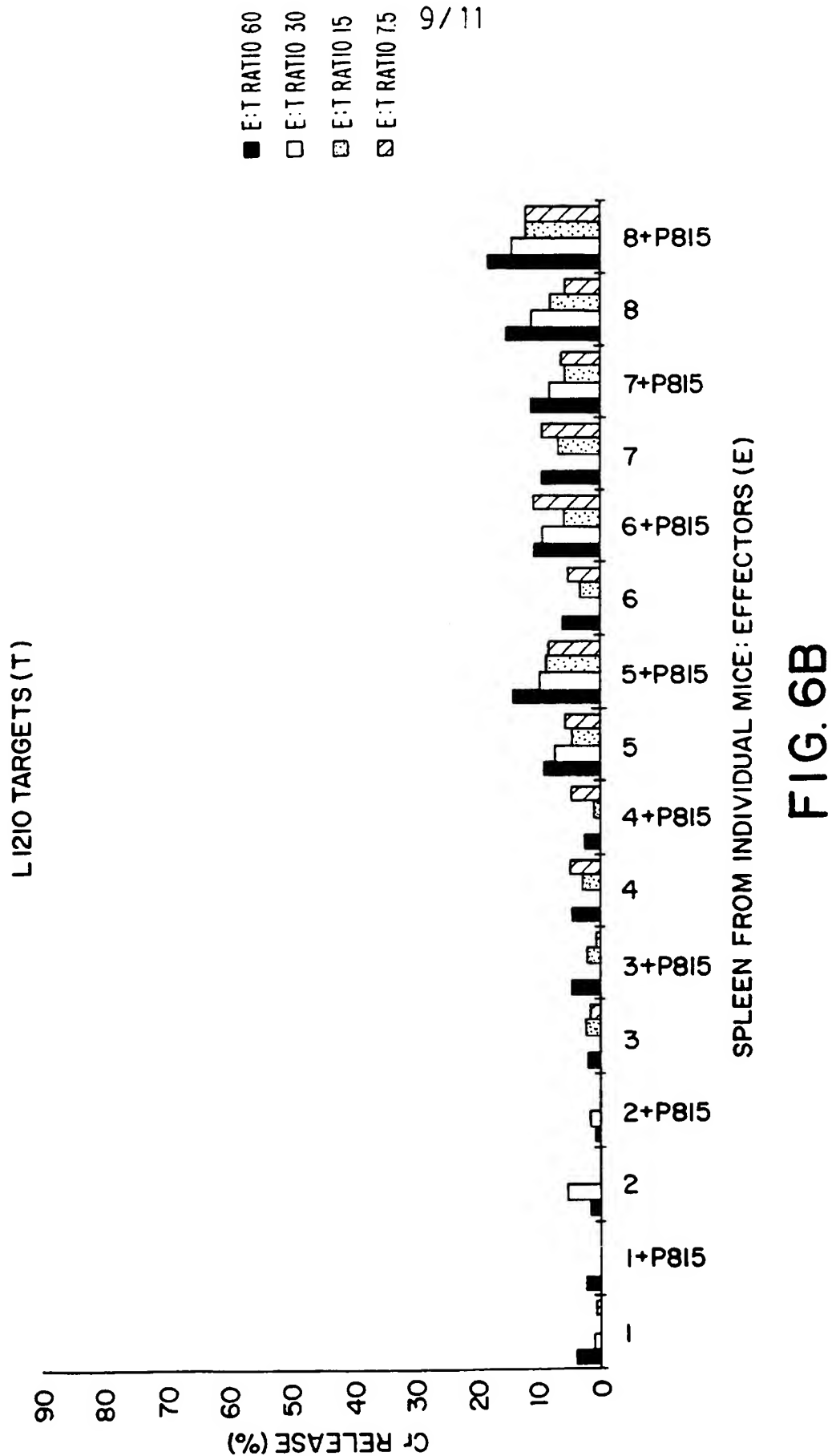


FIG. 5



**FIG. 6A**

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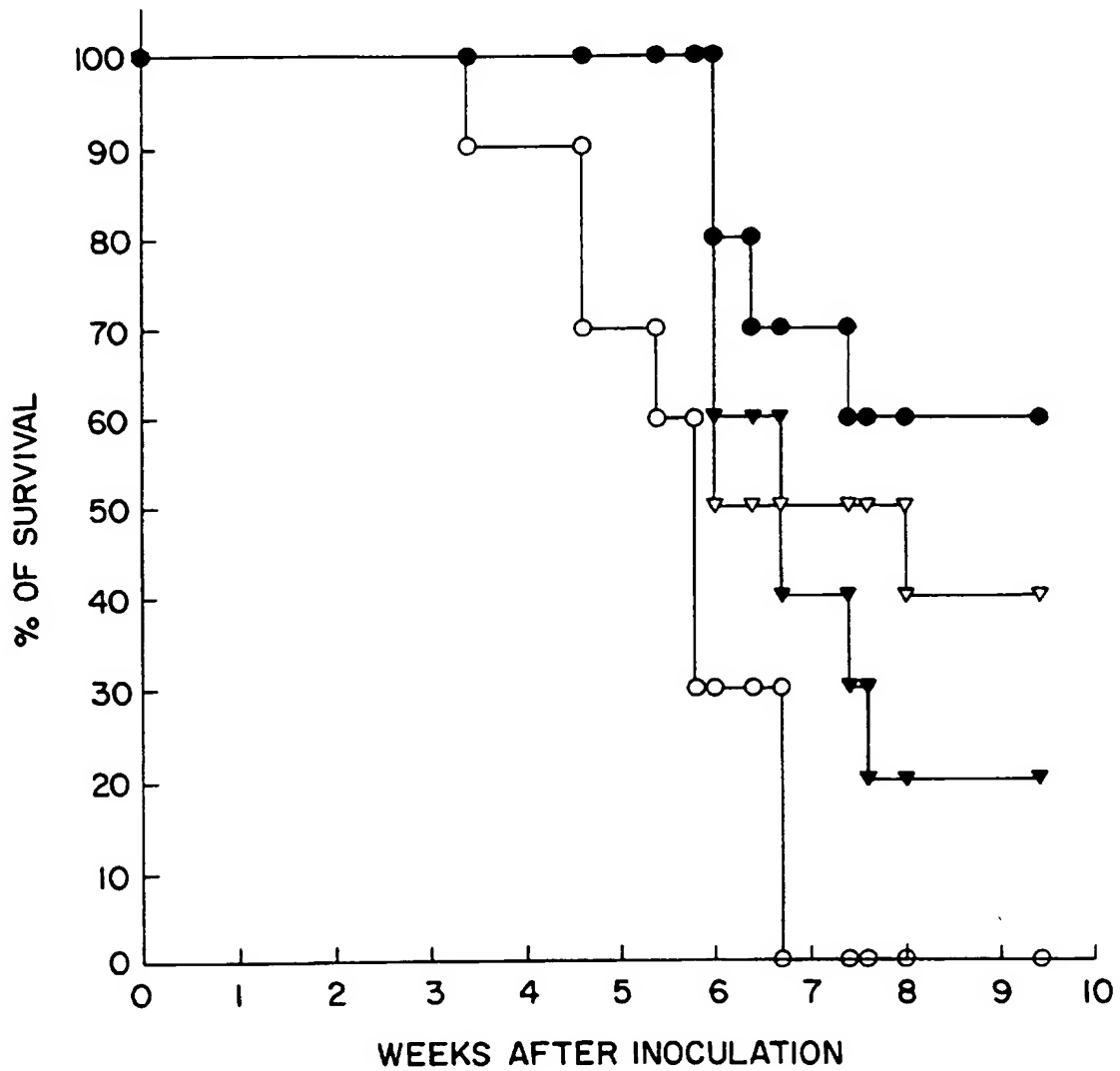


FIG. 7

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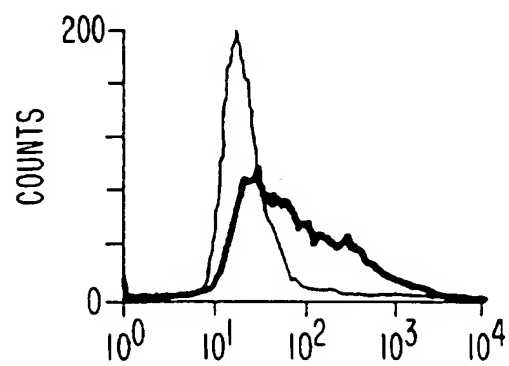


FIG. 8a

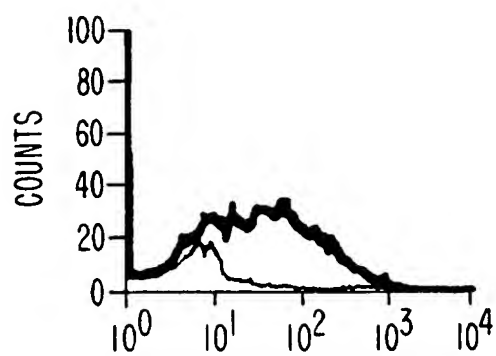


FIG. 8b

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/04370

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 35/14; C12N 5/22, 15/07

US CL : 435/172.2, 240.26; 424/93.7

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.2, 240.26; 424/93.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, Dialog, Medline, Biosis

cell fusion/cell hybrids/dendritic cells/tumor antigens/antigen presentation

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 263, Issued January 28 1994, Guo et al., "Effective Tumor Vaccine Generated by Fusion of Hepatoma Cells with Activated B Cells", pages 518-520, See entire document.	1-27
Y	Jornal of Eexperimental Medicine, Volume 178, Issued December 1993, Paglia et al., "Immortalized Dendritic Cell Line Fully Competent in Antigen Presentation Intiates Primary T Cell Responses IN VIVO", Pages 1893-1901, See entire document.	1-27



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

21 MAY 1996

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/04370

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Jornal Clinical Investigation, Volume 85, Issued March 1990, Markowitz et al., "Granulocyte-Macrophage Colony-Stimulating Factor Promotes Differentiation and Survival of Human Peripheral Blood Dendritic Cells IN VITRO", Pages 955-961, See entire document.	1-27
Y	Journal of Experimental Medicine, Volume 172 Issued August 1990, Inaba et al., "Dendritic Cells Pulsed with Antigens in vitro can Prime Antigen-Specific, MHC-Restricted T Cells in situ", Pages 631-640, See entire document.	22, 25
Y	Proceedings National Academy of Sciences, Volum 85, Issued August 1988, Hauser et al., "Activation and Expansion of Hapten- and Protein-Specific T Helper Cells from Nonsensitized Mice", Pages 5625-5628, See entire document.	23, 24, 26, 27
P,Y	Jornal of Experimental Medicine, Volume 183, Issued JanuaryN 1996, Zitvogel et al., "Therapy of Murine Tumors with Peptide-Pulsed Dendritic Cells: Dependence on T Cells, B7 Costimulation, and T Helper Cell 1-Associated Cytokines", Pages 87-97, See entire document.	18
Y	The Journal of Immunology, Volume 146, Issued May 15 1991, Grabbe et al., "Tumor Antigen Presentation by Murine Epidermal Cells", Pages 3656-3661, See entire document.	18
A	Annual Review of Immunology, Volume 9, Issued 1991, Steinman, "The Dendritic Cell System and its Role in Immunogenicity", Pages 271-296, See entire document.	1-27
A	Journal Experimental Medicine, Volume 176, Issued December 1992, Inaba et al., "Generation of Large Numbers of Dendritic Cells from Mouse Bone Marrow Cultures Supplmented with Granulocyte Colony-Stimulating Factor", Pages 1693-1702, See entire document.	1-27
A	Jornal Experimental Medicine, Volume 175, Issued May 1992, Inaba et al., "Identification of Proliferating Dendritic Cell Precursors in Mouse Blood", Pages 1157-1167, See entire document.	1-27



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 5/02</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/33265</b> <b>(43) International Publication Date:</b> 24 October 1996 (24.10.96)
<b>(21) International Application Number:</b> PCT/US96/05607 <b>(22) International Filing Date:</b> 22 April 1996 (22.04.96)  <b>(30) Priority Data:</b> 08/426,782 21 April 1995 (21.04.95) US  <b>(71) Applicants:</b> PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mount Auburn Street, Cambridge, MA 02138 (US). NEW ENGLAND DEACONESS HOSPITAL CORPORATION [US/US]; 1 Deaconess Road, Boston, MA 02215 (US).  <b>(72) Inventors:</b> JOHNSON, R., Paul; P.O. Box 9102, Southborough, MA 01772-9102 (US). ROSENZWEIG, Michael; P.O. Box 9102, Southborough, MA 01772-9102 (US). SCADDEN, David, T.; New England Deaconess Hospital, 21-27 Burlington Avenue, Boston, MA 02215 (US).  <b>(74) Agents:</b> GATES, Edward, R. et al.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).		<b>(81) Designated States:</b> JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IN VITRO DIFFERENTIATION OF CD34 <sup>+</sup> PROGENITOR CELLS INTO T LYMPHOCYTES  <b>(57) Abstract</b>  The invention involves a method for the <i>in vitro</i> T cell production. A monolayer of non-human primate thymic stromal cells are cocultured <i>in vitro</i> with primate hematopoietic T cell progenitor cells. This results in the differentiation and growth of mature T cells. The T cells may be isolated at any sequential stage of differentiation and further expanded by coculture with a mitogenic agent. The T cells also may be genetically altered at any stage of the process. The effect of agents on the growth and differentiation of T cells may be measured by comparing a coculture containing the agent with a control coculture and comparing the differentiation or growth of the T cells progenitor cells in the test culture with the control culture. Kits and novel populations of T cells are provided.		



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## IN VITRO DIFFERENTIATION OF CD34<sup>+</sup> PROGENITOR CELLS INTO T LYMPHOCYTES

5 Field of the Invention

The invention pertains to the growth and differentiation of T cells, and the genetic alteration of such T cells.

## **Background of the Invention**

10 Lymphoid differentiation of hematopoietic progenitor cells is dependent on interactions of these cells with thymic tissue. The prerequisite for these interactions has hindered the development of an *in vitro* system of lymphocyte differentiation. Lymphocytes originate from pluripotent stem cells that originate in the fetal liver and bone marrow. T lymphocyte differentiation normally occurs via a series of discrete developmental stages involving an initial  
15 primitive progenitor cell without lymphocyte specific cell surface markers (CD34<sup>+</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>), followed by acquisition of lymphocyte specific markers and loss of CD34 (CD34<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>), followed by differentiation into mature CD3<sup>+</sup> T cells expressing either CD4 or CD8 (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> or CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup>). Hematopoietic stem cells that are self renewing and pluripotent constitute approximately 1% of low density nucleated bone marrow  
20 cells. These cells express a high level of CD34 antigen on their surface, and as these pluripotent cells develop and commit to either the lymphoid, monomyeloid or erythroid cell series, the level of CD34 decreases.

Because T lymphocyte differentiation requires the interaction of precursor cells with thymic tissue, efforts to study this process in the laboratory have been quite limited. The study of human lymphopoiesis has been largely confined to the SCID-hu mouse *in vivo* model, thymic explant studies and human thymic monolayers. However, these existing techniques all share some significant drawbacks, among them a relatively low efficiency of T cell differentiation, the limited number of cells available for functional studies, and the difficulty in obtaining a readily available and consistent source of thymic tissue.

30        Recent progress using gene therapy to treat diseases involving T lymphocytes, including AIDS, has fostered increased interest in the development of laboratory techniques that allow *in vitro* evaluations of potential genetic therapies for these disorders. Existing techniques have not permitted detailed evaluation of the properties of candidate therapeutic genes in T lymphocytes derived from genetically modified progenitor cells. Techniques such as the SCID-hu mouse yield

only a small number of transduced T cells in mouse tissue. Extremely sensitive techniques such as polymerase chain reaction are necessary to detect foreign genes in these cells and sufficient numbers are not available to allow assessment of potential toxicity or to examine whether the therapeutic gene has had the desired effect, i.e. production of an enzyme or protection of the T cell against HIV infection.

We describe a system using non-human primate thymic derived cells that provides the appropriate conditions for *in vitro* T cell differentiation of human and non-human primate hematopoietic progenitor cells. T lymphocytes derived from these cultures respond normally to a variety of stimuli and express the diversity expected of mature T cells. This system provides significant advantages over existing techniques in that it: 1) replicates the complex process of T lymphocyte differentiation more closely than existing techniques, as evidenced by the normal progression of thymocyte stages observed at successive time points *in vitro* and the diverse representation of T cell receptors in T cells derived from these cultures; 2) provides a relatively large number of T cells necessary for laboratory analysis and therapeutic use including *in vitro* testing of potential gene therapy strategies and reinfusion into subjects *in vivo*; and 3) provides the capability to support *in vitro* T cell differentiation using a reagent that is available in relatively large amounts, thereby minimizing inter assay variation and enhancing quality control. Existing thymic culture techniques, particularly those involving SCID-hu mice, suffer from significant experiment to experiment variation. In contrast, since between  $10^9$  and  $10^{10}$  cells (sufficient for up to 10,000 assays) may be obtained and cryopreserved from a thymus harvested from a single animal, a large quantity of cells may be stored and used for multiple experiments.

### Summary of the Invention

According to one aspect of the invention, a method for *in vitro* T cell production is provided. The method involves coculturing *in vitro* a monolayer of non-human primate thymic stromal cells with primate hematopoietic T cell progenitor cells.

Preferably the thymic stromal cells are derived from a member of the species Macaca mulatta or Macaca fascicularis, most preferably Macaca mulatta. The preferred progenitor cells are derived from a member of the species Homo sapiens.

The hematopoietic T cell progenitor cells or descendants thereof can be isolated from the monolayer of thymic stromal cells at any time during the sequential stages of differentiation of such cells. In particular, the progenitor cells or descendants thereof may be isolated from the

monolayer prior to the differentiation of a substantial portion of such cells into either CD3<sup>+</sup>4<sup>+</sup>8<sup>-</sup> or CD3<sup>+</sup>4<sup>-</sup>8<sup>+</sup> cells.

The hematopoietic T cell progenitor cells or decedants thereof also may be cultured with a mitogenic agent to stimulate expansion of the cells or decedents thereof. Such expansion can occur, optionally, isolated from the stromal monolayer and in a state of arrested differentiation, that is, growth without further differentiation. Optionally, isolated and expanded cells can be reintroduced into culture with a monolayer of thymic stromal cells to promote further differentiation. According to one preferred embodiment, the predominant cell type isolated for expansion is CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup>. It is likewise contemplated that fully differentiated cells (CD3<sup>+</sup>4<sup>+</sup>8<sup>-</sup> and CD3<sup>+</sup>4<sup>-</sup>8<sup>+</sup> cells) can be cultured with a mitogenic agent to stimulate the expansion of such cells. The preferred mitogenic agent is non-mitotic feeder cells such as irradiated peripheral blood mononuclear cells.

According to another aspect of the invention, the hematopoietic T cell progenitor cells are genetically altered T cell progenitor cells. Such genetic alteration can occur prior to coculturing the hematopoietic T cell progenitor cells with the thymic stromal cells or at any stage during the coculture of the hematopoietic T cell progenitor cells with the thymic stromal cells. The progenitor cells or decedents thereof then can be isolated.

According to another aspect of the invention, a method for testing the effect of an agent on cells is provided. A monolayer of non-human primate thymic stromal cells is cocultured *in vitro* with primate hematopoietic T cell progenitor cells in the presence of the agent. The differentiation or growth of the T cell progenitor cells, or decedents thereof, exposed to the agent then is compared to the differentiation or growth of control T cell progenitor cells or decedents thereof as a determination of the effect of the agent. The conditions of culture, including the cell types, expansion conditions, genetic alteration and the like, can be varied as described above.

According to another aspect of the invention, a kit is provided. The kit includes a container containing a cryo-preserved non-human primate thymic stromal cell suspension and instructions for coculture of a monolayer of the thymic stromal cells with primate (including humans) hematopoietic T cell progenitor cells. The preferred cells are as described above. Such kits may be shipped to remote locations for thawing, forming the stromal monolayer and coculturing to promote *in vitro* T cell growth and differentiation.

According to still another aspect of the invention, an article of manufacture is provided. The article of manufacture is a container containing a representative population of T cells derived by any of the foregoing methods. Preferably the representative population of T cells is derived by

the method of claim 1 and most preferably the representative population of T cells is derived by culturing cells isolated from the coculturing conditions of claim 1 and further culturing the isolated cells with a mitogen to stimulate the expansion of such isolated cells.

It thus is an object of the invention to provide methods permitting the *in vitro* growth and  
5 differentiation of T cells.

Another object of the invention is to provide a test system for evaluating the effects of agents on T cell growth and differentiation.

Another object of the invention is to provide methods for yielding *in vitro* high numbers of T cells.

10 Another object of the invention is to provide methods of providing *in vitro* high numbers of T cells arrested at various stages of development.

Another object of the invention is to provide methods for genetically altering T cells *in vitro*.

Another object of the invention is to provide methods for expanding T cells *in vitro* for  
15 reintroduction of such cells *in vivo*.

Another object of the invention is to provide *in vitro* T cell differentiation that results in a substantially full repertoire of T cell types and results in T cells that respond similarly to those isolated from a subject.

These and other objects of the invention will be described in greater detail below.  
20

### **Detailed Description of the Invention**

Thymic stromal cells are derived from the disaggregation of a piece of thymus tissue. Such cells according to the invention are capable of supporting *in vitro* T cell growth and differentiation. Thymic stromal cells may include, but are not limited to, all cell types present in  
25 the thymus which are not lymphocytes or lymphocyte precursors or progenitors, e.g., epithelial, mesothelial cells, dendritic cells and macrophages.

Thymic stromal cells provide the supporting microenvironment in the intact thymus for the differentiation of T cell progenitor cells to mature T cells. The microenvironment includes soluble and cell surface factors expressed by the various cell types which comprise the thymic  
30 stroma.

Thymic stroma cells may be obtained from the thymus of a non-human primate at any time after the organ has developed to a stage at which it can support the differentiation of T cells. In primates, this stage of thymic development is achieved during the second trimester. At this stage

of development the thymus can produce peptide hormones such as thymulin,  $\alpha_1$  and  $\beta_4$ -thymosin, and thymopoietin, as well as other factors required to provide the proper microenvironment for T cell differentiation. It is preferred that the stromal cells are derived from a non-human primate thymus during the third trimester of gestation or from a thymus of a non-human primate neonate.

- 5 During the mid to late third trimester, the thymus stromal microenvironment is fully capable of inducing the differentiation of T cell progenitor cells to mature T cells.

The non-human primate stromal cells can be derived from any non-human primate.

- Examples include: Aotus trivirgatus - owl monkey; Ateles geoffroy - spider monkey; Cebus albifrons - white and brown capuchin monkey; Callithrix jacchus - common marmoset monkey;
- 10 Cercopithecus aethiops - African green monkey; Galago crassicaudatus - Bushbaby monkey; Macaca arctoides - stump-tailed macaque monkey; Macaca cyclopis formosan macaque monkey; Macaca fascicularis - Crab eater macaque (cynomolgus) monkey; Macaca mulatta - rhesus monkey; Macaca nemistrina - pig tail monkey; Macaca sylvana - barbary macaque; Pan troglodytes - chimpanzee; Papio anubis - baboon; Papio cynocephalus - yellow baboon; Papio
- 15 hamadryads - chaema baboon; Papio doguera - Dog-faced baboon; Saguinus fuscicollis - tamarin monkey; Saguinus oedipus - Cotton top tamarin monkey; and Saimiri sciureus - squirrel monkey. A preferred non-human primate is Macaca fascicularis and the most preferred non-human primate Macaca mulatta. The cells are then grown as a "monolayer". A monolayer of thymic stromal cells is recognized by one of ordinary skill in the art as an *in vitro* thymic stromal culture having a
- 20 thickness of one or more cells. A monolayer of thymic stromal cells encompasses a non-confluent layer of stromal cells, a confluent layer of stromal cells having a thickness of a single cell, and a layer of stromal cells in which the cells are stacked on one another to a thickness of two or more cells. In all cases, such monolayers are formed of suspensions of cells disaggregated from their native tissue structure and organization, preferably into single cell suspensions, which then are
- 25 plated into a vessel and permitted to attach to a surface. It is preferred that the cells be grown to confluency.

It should be noted that the stromal cells may be cryopreserved for later use or for storage and shipment to remote locations, such as for use in connection with the sale of kits.

- Cryopreservation of cells cultured *in vitro* is well established in the art. Subsequent to isolation of
- 30 a cell sample, cells may be cryopreserved by first suspending the cells in a cryopreservation medium and then gradually freezing the cell suspension. Frozen cells are typically stored in liquid nitrogen or at an equivalent temperature in a medium containing serum and a cryopreservative such as dimethyl sulfoxide. Because of the large numbers of cells that can be obtained and

preserved, the invention thus provides a reagent that is available in relatively large amounts, particularly for study of human T cells, thereby minimizing inter-assay variation and enhancing quality control.

Monolayers of the foregoing type are used to stimulate the growth and differentiation of  
5 primate hematopoietic T cell progenitor cells. This is accomplished by coculturing the monolayer together with the progenitor cells. Contact is contemplated for the most efficient stimulation.

Hematopoietic T cell progenitor cells are those cells capable of differentiating into mature T cells. They may be committed to the T cell lineage or uncommitted. T cell progenitor cells as used herein therefor include pluripotent cells which are capable of self-renewal and differentiation  
10 into all myeloid and lymphoid cell lineages, including T cells. T cell progenitor cells may be isolated from sources including bone marrow, umbilical cord blood or peripheral blood mobilized stem cells. Peripheral blood mobilized stem cells are obtained from the peripheral blood of subjects who have been treated with chemotherapeutic agents and/or cytokines to increase hematopoietic progenitor cells circulating in peripheral blood. The preferred hematopoietic T cell  
15 progenitor cells are those derived from humans.

Progenitor cells at various stages of differentiation may be used, although it is preferable to use progenitor cells in very early stages of differentiation. For example, CD34<sup>+</sup>CD38<sup>-</sup> or CD34<sup>+</sup>CD38<sup>+</sup>CD2<sup>+</sup> progenitor cells have been used successfully. Hematopoietic stem cells that are self-renewing and pluripotent constitute approximately 0.01% of low density nucleated bone  
20 marrow cells. These cells express a high level of CD34<sup>+</sup> antigen on their surface and do not express CD38. These cells eventually develop and commit to lymphoid, monomyeloid or erythroid cells, including T cells. CD34<sup>+</sup> T cell progenitor cells are negative for the T cell markers CD3, CD4 and CD8. During differentiation to mature T cells, the progenitor cells pass through an intermediate stage during which the cells express CD3, CD4 and CD8 on the cell surface  
25 (CD4<sup>+</sup>CD8<sup>+</sup>: "double positive"). Immature T cells in this intermediate stage of differentiation lose expression of the CD34 cell surface marker. Mature T cells are CD34<sup>+</sup>CD3<sup>+</sup>, and are further characterized by the presence of only one of the cell surface markers CD4 and CD8. Mature T cells thus are either CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> ("single positive").

As will be seen in greater detail below in connection with the detailed examples, coculture  
30 of CD34<sup>+</sup>CD38<sup>-</sup> T cell progenitor cells with a thymic stromal monolayer results in a expansion in the number of cells and in the differentiation of such cells into all stages including "single positive" cells. The repertoire of T cells produced more closely resembles the repertoire occurring *in vivo* than can be achieved by any known prior art method. The quantities of cells produced according

to the present invention, particularly after expansion, also exceed by 40-100 times the known *in vitro* methods for generating T cells. The methods of the invention, nevertheless, are believed to result in populations of T cells which differ from those populations that can be isolated from any source *in vivo* in terms of the relative numbers of the various types of cells present. Therefore, novel populations of cells are provided according to the invention. Such novel populations are described in terms of "representative populations." A representative population of T cells derived according to the invention means a population of T cells which contains all of the subpopulation of T cells derived from the *in vitro* differentiation of T cell progenitor cells in coculture with thymic stromal cells and contains them in their relative amounts as results from such coculture. As such, a container containing such a representative population differs from any population previously isolated in terms of relative numbers of cell types.

It should be noted that the differentiation of T cells on the monolayer is sequential, and T cells at various stages of differentiation therefore can be isolated from the culture. For example, T cells that are CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup> predominant at certain times during the culturing period. In addition, cell sorting methodologies or sequential positive selection using magnetic beads may be used to further isolate such cells from other cell types. In particular it is possible to isolate T cell populations prior to their terminal differentiation into "single positive" cells. For example, at day 14 of coculture as described below in the examples, approximately 60% of the human cells are CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup>. At day 28, the CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup> can represent less than 20% of the human cells. Conversely, at day 14 CD4<sup>+</sup>8<sup>+</sup> cells can represent less than 25% of the human cells while at day 28 CD4<sup>+</sup>8<sup>+</sup> cells can represent as many as 65% of the human cells. Likewise, CD4<sup>+</sup>8<sup>+</sup> cells can be as low as 2% on day 14 and as high as 15% on day 28.

It has been discovered that when such T cells are isolated from the stromal monolayer at a particular stage of differentiation, further expansion, for example, using mitogenic agents as discussed below, results in further growth of the cells but not further differentiation. Thus, the invention permits the isolation and expansion of high numbers of cells at a particular stage of differentiation. Such cells may be examined, for example, to test what mRNA they are producing and which receptors they are expressing. The relative effect of drugs on cells at different stages of differentiation may be tested. Likewise, such cells may be genetically altered at any particular stage of differentiation. It is further noted that such cells can be added back into culture with the stromal monolayer, with further differentiation reinitiated. Thus, for example, cells can be brought to a certain stage of development, isolated and genetically altered, and then further differentiated (optionally). Uses for such cells are further described below.



As mentioned above, the invention contemplates the further expansion of the hematopoietic T cells progenitor cells or decedents thereof. This is accomplished using a mitogenic agent and can be simply for increasing the number of cells available for use by those skilled in the art. Expansion can occur together in the culture with the stromal cells or isolated  
5 from the stromal cells. If together with the stromal cells, then growth and differentiation will occur. If isolated from the stromal cells, then growth may occur without further differentiation.

A mitogenic agent is an agent capable of supporting the expansion of a population of hematopoietic T cell progenitor cells or descendants thereof when incubated or cultured with such cells. Mitogenic agents are well known in the art and include agents that stimulate or support the  
10 growth of T lymphocytes. These agents include lectins, such as concanavalin A and phytohemagglutinin, and anti-CD3 antibody used alone or in combination with anti-CD28 antibody. These cells may also be cultured in the presence of agents such as cytokines, including the interleukins IL-2, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12 and the flk ligand, and feeder cells.

Feeder cells encompass cells that are capable of supporting the expansion of  
15 hematopoietic T cell progenitor cells or descendants thereof. The support which the feeder cells provide may be characterized as both contact-dependent and non-contact-dependent. The feeder cells may secrete or express on the cell surface factors which support the expansion of the progenitor cells. One example of feeder cells is peripheral blood mononuclear cells. Other examples include splenocytes, lymph node cells and dendritic cells. Feeder cells also may be cells  
20 that would not ordinarily function as feeder cells, such as fibroblasts, which have been engineered to secrete or express on their cell surface the factors necessary for support of T cell progenitor cell expansion. Feeder cells may be allogeneic, syngeneic or xenogeneic with respect to the T cell progenitor cells.

Feeder cell are made non-mitotic by procedures standard in the tissue culture art.  
25 Examples of such methods are irradiation of feeder cells with a gamma-ray source or incubation of feeder cells with mitomycin C for a sufficient amount of time to render the cells mitotically inactive.

As mentioned above, the hematopoietic T cell progenitor cells, or decedents thereof, can be genetically altered. Genetic alteration of a hematopoietic T cell progenitor cell (or thymic  
30 stromal cell) includes all transient and stable changes of the cellular genetic material which are created by the exogenous addition of an agent. Examples of genetic alterations include any gene therapy procedure, such as introduction of a functional gene to replace a mutated or nonexpressed gene, introduction of a vector that encodes a dominant negative gene product, introduction of a

vector engineered to express a ribozyme and introduction of a gene that encodes a therapeutic gene product. Natural genetic changes such as the spontaneous rearrangement of a T cell receptor gene without the introduction of any agents are not included in this concept.

Many agents capable of altering the genetic material of a cell are known to one of skill in the art. Such agents include viral vectors, episomal plasmid vectors, stably integrating plasmid vectors, and artificial chromosome vectors. Viral vectors include those derived from retroviruses, adenoviruses, adeno-associated viruses, herpesviruses, and pox viruses. Viral vectors may be delivered as virus particles or by another delivery mechanism as described below. Plasmid vectors include those plasmids that contain cDNAs, genomic DNAs and chimeric non-natural DNAs including synthetic nucleic acids. Vectors may be designed to integrate at a specific location in the genome by insertion or homologous recombination, may integrate randomly or may remain in the nucleus as a stable episomal nucleic acid. All of the above vectors may contain one or more genes or nucleic acid sequences.

The delivery of such genetic alteration agents may be accomplished by encapsulation in a viral particle or a synthetic particle such as a liposome. Many other delivery techniques are known in the art, including immunoconjugation, transfection and particle bombardment. Genetic alteration agents also include mutagens, antisense, ribozymes and the like.

Genetic alteration can be performed at any stage of differentiation of the hematopoietic T cell progenitor cells or decedents thereof. For example, the genetic alteration can be made directly on the cells as isolated from the primate (e.g., bone marrow, umbilical cord, etc.) or on a subpopulation thereof. Likewise, the genetic alteration can be carried out at any stage of the coculture, including during the coculture, or on any isolate of the coculture. In one preferred embodiment, retroviral vectors are used to genetically alter the cells during the coculture process in which the cells are dividing and differentiating. This promotes stable integration of the provirus into the genome of the dividing cells. Also as mentioned above, cells that have been isolated from a stromal monolayer and genetically altered can be reintroduced into culture with a stromal monolayer after the genetic alteration. The foregoing procedures have many utilities, including expanding the population of genetically altered cells for study or for therapeutic purposes as more fully described below.

According to another aspect of the invention, a method for testing the effect of an agent on cells is provided. The coculture of the stromal monolayer and primate hematopoietic T cell progenitor cells is carried out in the presence of the agent. The growth and differentiation of the T cell progenitor cells, or decedents thereof, exposed to the agent then is compared to the growth

or differentiation of control T cell progenitor cells or decedents thereof as a determination of the effect of the agent. The cells utilized, the culture conditions, the length of time that the cells are cultured, the expansion conditions and the like can vary to the full extent as described above.

What differs in this embodiment is the addition of an agent to the culture and a comparison of growth and/or differentiation to a control culture, preferably subject to otherwise identical conditions, but without the agent. The effect of the agent on the growth and differentiation of T cells can then be determined in this manner.

The agent can be anything known to be or suspected of being capable of affecting the growth and differentiation of the T cells. Examples include synthetic chemical agents, biochemical agents, cells, extracts, homogenates, and the like. The agent may act directly on T cell progenitors or decendants thereof or indirectly via action on the stromal layer. Specific examples include mitogens, cytokines, promoters of differentiation, antimetabolics, ribozymes, antisense, genes and pathogens such as viruses and bacteria. The foregoing is intended to be non-limiting; those skilled in the art will know numerous examples of agents useful according to the methods of the invention.

As will be clear to those skilled in the art, the invention enables the production *in vitro* of high volumes of T cells at various stages of development. It also enables *in vitro* test systems for analyzing the effect of an agent on T cell growth and development. It further enables a system for the production of genetically altered T cells which can be used *inter alia* in *in vivo* applications. The following general non-limiting examples will illustrate various uses of the invention; other uses will be immediately apparent to those skilled in the art.

The invention facilitates the study of T cell differentiation and growth by providing for the sequential development of high numbers of T cells into various stages of T cell development. Genes that are turned on and off can be identified and cloned via, for example, subtractive hybridization. The repertoire of receptors expressed at various stages also can be elucidated. The role of the thymus on T cell differentiation can be further elucidated. Greater quantities of molecules that are characteristic of particular stages of T cell development are available according to the invention. In general, intra and inter cellular events of T cell differentiation can be studied utilizing the invention.

The invention also facilitates the study of exogenously induced genetic changes to T cells and progenitors, including changes that result from infection by pathogens, such as those resulting in infectious disease and those associated with cancer, and changes that result in gene transfer for gene therapy purposes. For example, the cells and events associated with HIV infection can be

studied more easily *in vitro*. Likewise, drug agents including gene therapy drug agents can be evaluated *in vitro* for their protective effect against HIV infection or progression to disease. In general, laboratory evaluation of genetic therapies for disease of T cells can be studied *in vitro*. Such disease include, adenosine deaminase deficiency, purine nucleoside phosphorylase  
5 deficiency, leukocyte adhesion deficiency, and other congenital immune deficiency disorders involving T lymphocytes.

Ex vivo production of genetically altered T lymphocytes for introduction into a patient also is provided by the invention. Although hematopoietic stem cell therapy is generally conceived as involving reintroduction of genetically modified stem cells back into a patient, there  
10 can be advantages to differentiating genetically modified progenitor cells into T lymphocytes *in vitro* prior to reinfusion. These advantages include avoiding potential adverse affects of therapeutic genes in hematopoietic cells other than T cells, avoiding the many toxicities associated with bone marrow transplantation, and using stem cells as a source of genetically modified T cells for patients in whom CD4 T cell counts are not sufficient to support ex vivo expansion in  
15 reinfusion. Appropriate "education" of these *in vitro* derived T cells to recognize foreign antigens in the context of self major histocompatibility molecules and to avoid recognition of self (auto immunity) could be provided by the coculture of donor cells (e.g. fibroblasts or macrophages) with the rhesus thymic stroma culture. These cells could then be expanded by restimulation with feeder cells and reinfused. As an alternative technique, genetically modified cells could be  
20 removed from the monolayer at an immature stage prior to "education" (e.g. at the CD3+CD4+CD8+ stage) and reinfused into a patient where they would complete T cell maturation *in vivo*.

In general, the invention provides a method for expanding and differentiating cells *in vitro*, which cells then can be used for immune supplementation, necessary, for example, as a result of a  
25 genetic immune deficiency, aggressive treatment such as chemotherapy or radiation therapy and the like. In this regard, a subject's own cells can be removed, expanded and/or differentiated to a particular stage, as desired, and optionally genetically altered as discussed above. The cells then can be reintroduced into the subject at an appropriate time.

The invention also provides facilitated laboratory evaluation of drugs and cytokines that  
30 affect growth or differentiation of T cells, including those designed to enhance T cell production. Development of biologic agents to enhance production of specific hematopoietic cell lineages have been dependent on effective *in vitro* assays. Present assays are not suitable for testing large numbers of compounds for activity in enhancing the lymphopoiesis. However, given the

reproducibility of the nonhuman primate thymic stroma culture system and the availability of large quantities of tissue, this system would be ideally suited for such large scale screening. Although it is possible that there may be some differences in the effects of the agents on, for example, human and rhesus tissue, because of the phylogenetic similarity of these species and the fact that multiple human cytokines are known to be active in rhesus tissue, it is likely that these interspecies differences will be minimal.

The invention may also facilitate *in vitro* transduction of hematopoietic stem cell or T progenitor cells. Transduction of cells using viral vectors generally requires cell division, and determination of the appropriate conditions for cell division *in vitro* remains a significant challenge for the development of gene therapy. Stimulation of cell division with cytokines can lead to terminal differentiation of cells and loss of pluripotency. Transduction of cells cultured on rhesus thymic stroma is likely to mimic natural stimuli for cell division and may allow more optimal introduction of foreign genes into lymphocyte progenitors.

As mentioned above, the invention also provides kits. The kits include a cryopreserved suspension of non-human primate thymic stromal cells together with instructions for coculturing such cells with primate hematopoietic T cell progenitor cells, preferably human cells.

## **EXAMPLES**

### **Materials and Methods**

#### ***Animals***

Rhesus monkeys (Macaca mulatta) used in this study were normal, colony born animals maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Guide for the Care and Use of Laboratory Animals (DHHS Publication N. [NIH]85-23, revised 1985). For bone marrow aspirates animals were anesthetized with ketamine HCl and received lidocaine *in situ*.

#### ***Isolation of mononuclear cells from bone marrow and cord blood***

Bone marrow was extracted from mature rhesus macaques using an Illinois sternal/iliac aspiration needle (Stuart, USA) and a heparinized syringe. One to three samples with volumes of 1 to 8 ml were aspirated per iliac crest.

Heparinized bone marrow was obtained from normal human volunteers who provided written informed consent to a New England Deaconess Hospital Institution Reviewed Boards approved protocol.

Five milliliters of venous cord blood was extracted using a heparinized syringe prior to the severing of the umbilical cord during a Caeserean section delivery of a Rhesus macaque. After the umbilical cord was severed and the infant extracted, the placenta was removed by clamping the umbilical vein proximally and severing distally to the placenta. Immediately after the placenta was removed the umbilical vein was unclamped and the blood contained in the placenta drained into an appropriate heparinized container. Before processing, the cord and placenta blood was mixed together.

After extraction the bone marrow or cord/placenta blood was diluted 2:1 with washing media (RPMI 1640, 10 IU/ml penicillin, 10  $\mu$ g/ml streptomycin, 1 mM L-glutamine). The sample(s) were then underlayered with a volume of Ficoll-Hypaque (1.077 g/ml) equal to half of the diluted sample volume so that a distinct sample/Ficoll interface formed. After centrifugation for 45 minutes at 400 X g the interface containing mononuclear cells was removed. The cells were then washed by resuspending in culture medium and centrifuging for 10 minutes at 400 X g. The resulting pellet was resuspended in 6 ml of ammonium chloride lysing buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 1.0 mM  $\text{KHCO}_3$ , 0.1 M  $\text{Na}_2\text{EDTA}$ ) for 3 minutes to lyse any remaining erythrocytes. The suspension was then diluted with media and washed twice more. After the final wash the cells were resuspended in 1-2 ml media and the number of viable cells was determined by trypan blue exclusion.

#### *Isolation of CD34 positive cells*

Cells expressing the surface antigen CD34 were isolated using the Dynal CD34 Progenitor Cell Selection System (Dynal, Lake Success, New York). The mononuclear cells isolated from bone marrow or umbilical cord blood were suspended in isolation buffer (PBS, 2% heat inactivated fetal bovine serum, 10 IU/ml penicillin, 10  $\mu$ g/ml streptomycin) at a concentration of  $2.5 \times 10^7$  cells/ml. The suspension was then added to magnetic anti-human CD34 beads that crossreact with rhesus CD34 (Dynal M-450 CD34) in a ratio of  $4.0 \times 10^7$  beads per ml of suspension, in a round bottom tube. (Dynabeads M-450 CD34 are superparamagnetic beads bound to monoclonal antibody specific for CD34.) The mixture was vortexed gently and incubated at 4°C for 45 minutes with gentle tilt rotation using a Dynal Sample Mixer. After incubation the bead/cell mixture was resuspended in a larger volume of isolation buffer and placed

in a magnetic separation device for 2 minutes to allow the cell/bead complexes to accumulate to the tube wall. While still exposed to the agent, the suspension containing the cells not bound to the magnetic beads was aspirated. The cell/bead complexes were washed three more times in this manner, pooling the suspensions containing the CD34 negative cells into the same tube. The tube  
5 containing the released cells (CD34<sup>-</sup>) was then placed on the magnetic separator to remove any remaining beads and this supernatant was transferred to a new conical tube. All CD34<sup>+</sup> cells attached to beads were washed twice in a minimum of 10 ml of isolation buffer with centrifugation at 2000 rpm for 8 min. Cells bound to magnetic beads were then resuspended in 100  $\mu$ l of isolation buffer per  $4 \times 10^7$  beads used, with a minimum volume of 100  $\mu$ l. The CD34  
10 positive cells were then detached from the beads by adding an equal volume of an anti-idiotypic antibody (DETAChA<sup>®</sup>BEAD CD34, Dynal), vortexing, and gently mixing at room temperature using a Dynal Sample Mixer for one hour. The cells were isolated from the cell/bead suspension by adding isolation buffer and placing the tube in the magnetic separation device for 2 minutes. After the beads migrated to the tube wall, the supernatant containing the CD34 positive cells was  
15 transferred to a new tube. The beads were washed three more times with the suspensions containing the released cells pooled into the same tube. The tube containing the released CD34<sup>+</sup> cells was then placed on the magnetic separator to remove any remaining beads, and the supernatant was transferred to a new conical tube. The cells were washed twice in a minimum of 10 ml of isolation buffer with centrifugation at 2000 rpm for 10 minutes.

20

#### ***Blast forming unit/Colony forming unit (BFU/CFU) assays***

After isolation, the bone marrow or cord/placenta mononuclear cells were diluted to a concentration 10-fold greater than the final desired plating concentration in plating media (DMEM with 2% heat inactivated fetal bovine serum). The final plating concentrations were:  
25 bone marrow,  $1 \times 10^6$  cells/ml; cord/placenta blood,  $1 \times 10^5$  cells/ml; and CD34 positive cell,  $1 \times 10^4$  cells/ml. The following contents were then added to a 50 ml conical centrifuge tube for a total volume of about 3.15 ml: Iscove's DMEM with 10 IU/ml penicillin/10  $\mu$ g/ml streptomycin/1 mM L-glutamine, rEPO (1 U/ml), rGM-CSF (1 ng/ml), rIL-3 (10-20 ng/ml), cell suspension (0.3 ml), and methyl cellulose (2.8 ml, Stem Cell Technologies, Vancouver, BC, Canada). The mixture  
30 was gently vortexed and allowed to stand for several minutes to remove air bubbles. The cell mixture was then added in duplicate to 35 mm cell culture dishes with grids with a syringe and blunt needle. After dispensing the mixture, any bubbles present were removed with the syringe. The duplicate plates were placed inside a 100 mm cell culture dish along with 3 ml sterile PBS to

prevent desiccation. The plates were incubated in a 37°C incubator with 5% CO<sub>2</sub>. After two weeks the number of erythroid colonies and granulopoietic colonies were counted under a microscope. The erythroid colonies were distinguishable from the granulopoietic colonies based on the presence of a red hue caused by the hemoglobin contained in each cell. Appropriate conversions were made to the cell counts to account for the differences in starting dilutions.

### *Thymic monolayer cultures*

Thymic tissue from third trimester (120-165 days, 165 days being full term) Rhesus fetuses delivered by Caesarean section was minced into small fragments (1 mm<sup>3</sup>) using blunt nosed scissors. The fragments were then digested into a single cell suspension by incubation in PBS with 0.5 mg/ml of collagenase (Sigma-C9407) and 2 units/ml DNaseI (Sigma-DN-25) at 37°C for 60 minutes with frequent agitation. The solution was pipetted vigorously to break up any incompletely digested fragments. The number of viable cells was counted using a hemacytometer based on trypan blue exclusion by the viable cells. The cell suspension was washed once in culture media (RPMI 1640, 10% heat inactivated fetal bovine serum, 10 IU/ml penicillin, 10 µg/ml streptomycin, 1 mM L-glutamine) followed by centrifugation at 2000 rpm for 10 minutes. Cryopreservation of the thymic stromal cells was performed using a freezing solution of 90% fetal bovine serum and 10% dimethylsulfoxide. Thymic stromal cells used after cryopreservation and thawing fully supported the growth and differentiation of CD34<sup>+</sup> cells. The suspension was placed into 24 well cell culture plates at a concentration of 5 x 10<sup>5</sup> to 1 x 10<sup>6</sup> cells per well in a volume of 2 ml culture medium. After two days in a 37°C incubator with 5% CO<sub>2</sub> the nonadherent cells were removed and the adherent cell layer was washed three times with culture media to remove any loose cells. The monolayer was then maintained in culture media which was changed at least twice per week. After 6 days in culture, CD34 positive cells were added to the monolayer at concentrations ranging from 10<sup>3</sup> to 10<sup>5</sup> cells per well. After 14 days, cells were stimulated on the monolayer, using either 5 µg/ml Con A (concanavalin A, Sigma) or anti-rhesus CD3 (30 ng/ml) (6G12, provided by Dr. Johnson Wong; other anti-rhesus CD3 antibodies are commercially available or may be prepared by one of ordinary skill in the art) and anti-CD28 (30 ng/ml; Immunotech, Westbrook, ME) in combination with 1 x 10<sup>6</sup> irradiated (3000 Rads) human peripheral blood mononuclear cells (PBMC) and 100U/ml recombinant human IL-2 (Dr. Maurice Gately, Hoffman-LaRoche; may also be obtained from commercial sources). Cells may optionally be removed from the monolayer after 7 days and stimulated for expansion as described above.



### *Cells*

Bone marrow stroma cultures were established in 24 well plates as described by Sutherland and Eaves (Culture of Hematopoietic Cells, pp. 136-162, 1994). Skin derived fibroblasts were obtained from skin biopsy samples that were minced into 1-2 mm pieces. These  
5 pieces were placed in a scored cell culture dish, covered with a sterile microscope slide, and incubated with growth medium containing DMEM with 20% FCS, 10 mM Hepes, 10 IU/ml penicillin, 10 mg/ml streptomycin, 10 µg/ml vancomycin and 10 µg/ml gentamycin. A confluent layer of fibroblasts was obtained after 10-14 days. Cells were removed from the plate by incubating in trypsin(0.05%)/EDTA (0.53 mM) for 2 minutes at 37°C. Cells were washed and  
10 replated in 24 well tissue culture plates at a concentration of  $6 \times 10^4$  cells/well. Confluent cell layers were obtained in 3-4 days and bone marrow derived CD34<sup>+</sup> cells were added to these wells.

### *Fluorescent antibody analysis*

15 The distribution of CD3, CD4 and CD8 and other proteins on the differentiated CD34 positive cells removed from thymic monolayers was determined at varying stages of cell maturity by antibody binding and FACS analysis. Antibodies used for immunophenotyping rhesus and human cells included anti-CD3 (6G12, rhesus specific; Kawai et al. Transplant Proc., 26:1845-1846, 1994), anti-CD4 (OKT4) (Ortho Diagnostics, Raritan, NJ), anti-CD8 (Leu-2a) (Becton-  
20 Dickinson, San Jose, CA), anti-CD16 (Leu-11A) (Becton-Dickinson, San Jose, CA), anti-CD28 (Immunotech, Westbrook, ME) and anti-CD34 (QBend-10) (Immunotech, Westbrook, ME). An antibody specific for human CD3 (Leu-4) (Becton-Dickinson) was used in the xenogeneic studies. All antibodies used in the experiments were specific for human markers and cross-react with rhesus markers except the anti-CD3 (6G12) antibody which is specific for rhesus CD3. The  
25 presence of CD4 was detected by staining with anti-CD4 antibody (OKT4) directly conjugated to phycoerythrin (PE) (Ortho Diagnostics, Raritan, NJ). The presence of CD3 was detected by staining with anti-CD3 antibody directly conjugated to FITC using the Quick Tag FITC Conjugation Kit (Boehringer Mannheim, Indianapolis, IN). The presence of CD8 was detected by staining with biotinylated anti-CD8 (Leu-2a) antibody (Becton-Dickinson, San Jose, CA).  
30 Streptavidin conjugated to the fluorescent dye Red 670 (GIBCO-BRL, Frederick, MD) was used as a detection reagent for biotinylated anti-CD8 antibody. Cells were stained in the presence of staining media (PBS with 2% heat inactivated fetal bovine serum). After antibody staining the cells were either analyzed while viable in staining media or fixed with freshly prepared 2%

paraformaldehyde. Three color flow cytometry analysis of the thymocytes was performed using a FACScan flow cytometer (Becton Dickinson).

#### ***Limiting dilution assay***

5 Thymic monolayer cultures were established in 96 well plates as described above using  $5 \times 10^4$  thymic stroma cells per well. Purified CD34<sup>+</sup> bone marrow derived cells were added to the 96 well plate in serial dilutions from 1000 to 1 cell per well in 24 well replicates for each serial dilution. Cultures were maintained as described above. Cell growth was evaluated after 14 days of culture by counting viable cells, using the criterion of a doubling of input cell number to  
10 establish growth. Growth of T cells in wells scored as positive based on cell numbers was confirmed by FACS analysis of cells; all wells analyzed (>10) confirmed growth of T lymphocytes in positive wells. Data was generated on a complimentary log-log plot assuming the number of positive wells follows a binomial distribution, and that the number of cells in any well follows a Poisson distribution. In wells with more than 1000 cells, immunophenotyping for CD3, CD4 and  
15 CD8 was performed as described above.

#### ***PKH26 labeling of CD34 cells***

Purified CD34<sup>+</sup> cells ( $1 \times 10^6$  cells) were transferred to a 15 ml polypropylene conical tube and washed once in medium without serum at 25°C. After centrifugation, cells were  
20 resuspended in 25°C of serum free medium and rapidly mixed with PKH26 ( $4 \times 10^6 \mu\text{M}$ ; Sigma) for 5 minutes at room temperature. The labeling reaction was stopped by the addition of 10% fetal bovine serum. Cells were washed three times and analyzed by fluorescence microscopy (546 nm excitation, Texas red filter cube) and flow cytometry (FACScan, Becton Dickinson) for efficiency of labeling. Labeled CD34<sup>+</sup> cells were added to monolayer cultures as described above.

25

#### ***mRNA extraction and cDNA synthesis***

Messenger RNA was extracted from cells grown on a thymic monolayer harvested on a weekly basis over a four week period (Days 0,7,14,21,28). The extraction was performed using guanidinium thiocyanate and oligo dT spun columns (QuickPrep Micro mRNA Purification Kit;  
30 Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. mRNA samples were stored at -70°C. The first strand cDNA was synthesized in a 40  $\mu\text{l}$  final volume, using approximately 2  $\mu\text{g}$  of mRNA, 1  $\mu\text{g}$  of random primer, and 6.25 units of AMV reverse transcriptase

(GIBCO/BRL). Samples were incubated for 10 minutes at room temperature, 1 hour at 42°C, 5 minutes at 95°C, and 5 minutes at 4°C.

#### ***RT-PCR for RAG-2 gene expression***

5 cDNA was prepared by reverse transcription using random primers and Moloney MuLV reverse transcriptase (GIBCO-BRL, Grand Island, NY). cDNA was amplified using primers specific for a 415 bp region of the human RAG-2 gene, which is expressed transiently only by cells undergoing lymphocyte differentiation. The oligonucleotide primers were synthesized on a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). The sequences of the  
10 RAG-2 primers were as follows: 5' primer: GTC CCG GGC GCT GCA (669-683; SEQ.I.D.NO. 1), 3' primer: CCT CCC ACA CGC TTG CAG T (1082-1064; SEQ.I.D.NO. 2) and probe: CGT TGG GTC GGT GTC AGC CAC TCT CAC CTC CC (701-733; SEQ.I.D.NO.3). One quarter of the cDNA product was added to each PCR reaction (final volume 50 µl) with 50 pmole of each oligonucleotide primer and 2.5 U Taq DNA Polymerase (Pharmacia). PCR amplification  
15 was performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norwalk, CT) for 35 cycles of denaturation at 94° C for 30 s, annealing at 60° C for 30 s and extension at 72° C for 30 s. PCR products were separated on 2% agarose gels containing ethidium bromide and photographed under UV light. Specificity was confirmed by Southern blot hybridization with a <sup>32</sup>P-labelled 32-nucleotide RAG-2 probe. β-actin primers (Clontech, Palo Alto, CA) were used to amplify the  
20 cDNA as a control.

#### ***RT-PCR analysis of TCR VB gene expression***

Expression of the 25 Vβ gene subtypes was determined by RT-PCR. A PCR reaction mixture (45µl) containing 1.5mM MgCl<sub>2</sub>, 0.100mM of each dNTP, 2.5 units of Taq polymerase,  
25 and 27.0 pmol of the antisense Cβ primer was aliquoted from a master mix into 0.2 ml Microamp tubes (Perkin Elmer) each containing 27.0 pmol of each Vβ primer (VB1-24) (Chen et al., J. Immunol., 151:2177-2187, 1993). cDNA from each time point (5µl) was aliquoted into each 45µl reaction tube. In addition two controls were run simultaneously in separate reaction tubes for each time point : Cβ primers as an internal control , and β-Actin to confirm the integrity of  
30 the cDNA. A water control containing no cDNA was also run with each set of reactions to rule out contamination. PCR amplification was performed in a Gene Amp 9600 thermal cycler. Reaction conditions were a modified hot start followed by 30 cycles of 94°C, 55°C and, 72°C for

1 minute each. PCR products were run on a 1.5% agarose gel containing ethidium bromide and photographed under UV light.

#### **Example 1: Immunophenotyping of rhesus bone marrow**

5        The surface phenotype of freshly isolated rhesus bone marrow was determined by staining with fluorescent antibodies and subsequent FACS analysis. Approximately 30% of all bone marrow mononuclear cells expressed CD34 and the level of CD34 expression varied markedly in this population from CD34<sup>lo</sup> to CD34<sup>hi</sup>, with the majority of cells expressing low to intermediate levels of CD34. Approximately 1-4% of the CD34<sup>+</sup> cells express high levels of this  
10 epitope, and these are presumed to be enriched for the uncommitted or pluripotent hematopoietic progenitor cells. Cells isolated by immunomagnetic separation were 95% pure for CD34 expression as determined by flow cytometry, and were greatly enriched for the population of progenitors expressing high surface levels of CD34.

Analysis of purified CD34<sup>+</sup> cells did not reveal any cells with the phenotype of mature or  
15 immature T lymphocytes. A small percentage of CD34<sup>+</sup> cells were shown to express CD4. A variable percentage of CD34<sup>lo</sup> cells expressed high levels of CD2, but these cells were negative for CD3 expression. A small discrete population of CD34<sup>+</sup> cells were found to coexpress CD8. However, neither the CD2<sup>+</sup>, CD4<sup>+</sup> or CD8<sup>+</sup> subpopulations were shown to coexpress CD3, indicating the absence of a T cell receptor complex and demonstrating the absence of a significant  
20 number of contaminating T cells.

#### **Example 2: Immunohistochemistry of thymic stromal cells**

Immunohistochemistry was performed on thymic monolayers, and determined that a heterogeneous population of cells was present. Positive staining was detected with: antibodies  
25 directed against desmin and vimentin, indicating myofibroblasts; antibodies directed against cytokeratins, indicating epithelial cells; and antibodies against CD68 and CD14 indicating macrophages. In addition expression of both MHC class I and MHC class II was detected. The structure of the adherent cell layer was described as non-uniform with discrete raised areas that exhibit the greatest staining for antibodies directed against macrophage epitopes, epithelial cells  
30 and MHC class II. The thymic cells thus appear to develop some form of three dimensional structure in culture.

**Example 3: Differentiation of CD3<sup>+</sup> T lymphocytes from CD34<sup>+</sup> cells on rhesus thymic monolayers**

In order to assess the ability of an *in vitro* system to support T lymphocyte differentiation, purified rhesus bone marrow or cord blood derived CD34<sup>+</sup> cells were cultured on rhesus thymic stroma monolayers. Wells inoculated with  $5 \times 10^4$  -  $1 \times 10^5$  purified CD34<sup>+</sup> cells showed an average expansion in cells to  $7.2 \times 10^5$  by day 14. On day 14 of culture, flow cytometric analysis revealed the presence of a discrete population of cells with a forward and side scatter profile characteristic of lymphocytes. By three color flow cytometry, these cells were found to consist of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> (double positive) cells characteristic of immature T lymphocytes and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> and CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> single positive T lymphocytes. After 21 days in culture, 96% of gated cells were CD3 positive and coexpressed either CD4 or CD8. Similar results were obtained with bone marrow or cord blood CD34<sup>+</sup> derived cells. Compared with day 14, these cells exhibited a more mature phenotype, with a decrease in the number of double positive cells, and an increase in the numbers of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> and CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> single positive thymocytes. Stimulation with either anti CD3 and anti CD28 antibodies or ConA in the presence of irradiated human peripheral blood mononuclear cells (PBMC) generated as many as  $3 \times 10^7$  CD3<sup>+</sup> single positive cells. A subpopulation of cells was observed that exhibited a higher forward and side scatter profile. FACS analysis demonstrated these cells to express the phenotype CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>lo</sup>CD16<sup>+</sup>. The surface level of CD3 in this population varied from low to intermediate. This phenotype is characteristic of natural killer cells.

**Example 4: CD3<sup>+</sup> Lymphocytes are derived from CD34<sup>+</sup> cells**

A number of studies were conducted to demonstrate that the T lymphocytes observed in culture were derived from the CD34<sup>+</sup> cells and dependent on thymic tissue. Control experiments included culture of: 1) CD34-depleted bone marrow on rhesus thymic stroma; 2) CD34<sup>+</sup> purified cells on bone marrow stroma; and 3) CD34<sup>+</sup> cells cultured on rhesus fibroblasts.

These conditions were universally negative for generating the appropriate milieu for CD34<sup>+</sup> cells to develop into T lymphocytes. These experiments demonstrated that the effects observed in culture were specific to the thymic monolayers, and dependent on CD34<sup>+</sup> cells. The only conditions that resulted in the development of both double and single positive thymocytes and natural killer cells was the addition of CD34<sup>+</sup> bone marrow derived cells to thymic monolayer cultures. This demonstrates that lymphoid progenitors are present in CD34<sup>+</sup> purified fractions of bone marrow.

Further evidence to demonstrate that the CD3<sup>+</sup> lymphocytes developed from CD34<sup>+</sup> bone marrow derived cells was provided by assays using PKH26 labeling of CD34<sup>+</sup> cells. We achieved close to 100% labeling of CD34<sup>+</sup> cells at the time of labeling, as determined by flow cytometry. The presence of PKH26 was observed in both double positive and single positive CD3<sup>+</sup> lymphocytes derived from PKH26 labeled CD34<sup>+</sup> cells grown on thymic monolayers. The level of cell membrane labeling with PKH26 was observed to decrease with the cells' maturity and time in culture. This was expected as PKH26 is incorporated stably into the membrane of the primary labeled cells; thus the amount of PKH26 per cell will decrease exponentially with each cell division. PKH26 labeled cells were shown to express CD3, CD4 and CD8 on their cell surface, demonstrating that the CD34<sup>+</sup> bone marrow derived cells had developed into T lymphocytes.

**Example 5: Estimation of lymphoid precursor frequency by limiting dilution assay**

A single stem cell has the ability to generate multiple progeny via the process of differentiation. We determined the precursor frequency of purified CD34<sup>+</sup> cells by quantification of the number of viable thymocytes and T cells that could be generated in thymic monolayer cultures. Data from representative limiting dilution assays determined the precursor frequency to be 1/93 (1/68 - 1/128), with a chi square of 12.3, for lymphocyte progenitors from CD34<sup>+</sup> bone marrow.

**Example 6: Differentiation of human CD34<sup>+</sup> cells into T cells on rhesus monolayers**

Parallel experiments examined the characteristics of the thymic monolayers in a xenogeneic system. Human bone marrow derived CD34<sup>+</sup> cells grown under the same conditions on rhesus thymic monolayers generated CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> and CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> cells. Flow cytometry analysis was performed with an anti-CD3 antibody that is specific for human CD3, which provides further data to demonstrate that the exogenously added CD34<sup>+</sup> cells develop into T cells in a thymic monolayer environment. A rhesus specific CD3 antibody failed to bind to the T cell progeny isolated from the monolayers, confirming the absence of rhesus T cells. The level of CD3 staining demonstrated tripartite distribution characteristic of thymocytes.

**Example 7: CD3<sup>+</sup> T lymphocytes derived from thymic monolayers are functional**

Cells derived from thymic monolayers were shown to be responsive to a number of proliferation signals characteristic of thymocytes and T cells. Our results show that from 14 days in culture, there is a population of cells present that respond to stimulation with lectin in the

presence of irradiated feeders, and this results in expansion of the cell numbers. Our data demonstrate that this method of stimulation employed every 14 days resulted in marked expansion of T cell progeny in culture, as shown in Table 1. The cells exhibited characteristics unique to thymocytes when stimulated with anti-CD3 antibody alone. In this situation, cell numbers decreased, demonstrating a poor response to anti-CD3. However, when anti-CD28 was provided as a costimulation, we observed significant expansion of the T cell population, with expansion of both double and single positive thymocytes. Anti-CD28 alone failed to yield a proliferative response.

Expt.	Day 0	Day 14	Stimulus	Day 28	Day 42	Day 56	Day 70
1	$0.1 \times 10^6$	$0.05 \times 10^6$	lectin	$2 \times 10^6$	$10 \times 10^6$	$16 \times 10^6$	$225 \times 10^6$
2	$0.1 \times 10^6$	$0.2 \times 10^6$	lectin	$2.1 \times 10^6$	$4 \times 10^6$	$64 \times 10^6$	$60 \times 10^6$
3	$0.08 \times 10^6$	$0.2 \times 10^6$	lectin	$4.5 \times 10^6$	$18 \times 10^6$	$16 \times 10^6$	$27 \times 10^6$
4	$0.1 \times 10^6$	$1.5 \times 10^6$	antiCD3/CD28	$3.2 \times 10^6$	$9 \times 10^6$	$99 \times 10^6$	$17 \times 10^6$
5	$0.1 \times 10^6$	$1.6 \times 10^6$	antiCD3	$0.08 \times 10^6$	$0.04 \times 10^6$	-	-
6	$0.1 \times 10^6$	$0.8 \times 10^6$	antiCD28	$0.05 \times 10^6$	-	-	-

Table 1: Response of T cells from thymic monolayer cultures to mitogenic stimuli.

Representative cell counts of samples generated from thymic monolayer cultures. Cell number is the total viable cell number as determined by trypan blue exclusion. Cells from experiments 1, 2 and 3 were stimulated with Con A every 14 days. Experiment 4 was stimulated with anti CD3 and anti CD28 antibodies at day 14 and then with Con A every 14 days. Experiment 5 was stimulated with anti CD3 alone at day 14, and then Con A at day 28. Cells were stimulated with indicated mitogens in the presence of irradiated human PBMC and 100 U/ml rIL-2. Numbers of cells selected time points reflect calculated total cell number based on the observed expansion of a subset of total cells.

The cultures remained responsive to either lectin or anti-CD3 and anti-CD28 restimulation for 6-12 weeks. Cultures could thus be maintained for protracted periods, and allowed the generation of large numbers of T cells from CD34<sup>+</sup> bone marrow derived cells.

#### Example 8: Temporal expression of lymphoid lineage genes

Commitment to lymphopoiesis is accompanied by a highly regulated process of differentiation, which is characterized by the temporal expression of various lymphoid lineage associated genes. Expression of the T lymphocyte associated gene products, namely TCR and

RAG-2, were determined at sequential time points by RT-PCR. The CD34<sup>+</sup> purified population was devoid of both TCR and RAG-2 gene products. At day 7 and 14 cells derived from the thymic monolayers were observed to express products of the RAG-2 gene. Expression of RAG gene products is required for rearrangement of the antigen receptor. Subsequent to day 14 the  
5 level of RAG-2 expression decreased. Simultaneous analysis of TCR gene products by RT-PCR detection demonstrated that TCR products were present subsequent to RAG-2 expression. TCR gene products were detected from day 14 onwards in cells derived from thymic monolayer cultures.

#### 10 Example 9: Analysis of TCR V $\beta$ Subsets

To further analyze the diversity of T cells that developed in this *in vitro* system, RT-PCR determination of TCR V $\beta$  subsets was performed at weekly intervals. Results were uniformly negative at both day zero (CD34<sup>+</sup> purified cells) and at day seven. Our results demonstrate that by day 14, 23 out of 25 V $\beta$  subsets were detectable by RT-PCR. At day 14 the cultures  
15 underwent stimulation with lectin and IL-2. Subsequent analysis of the RNA present at day 28 indicated the presence of 21 out of 25 TCR V $\beta$  subsets, suggesting that the lectin stimulation may result in selective expansion of certain V $\beta$  subsets.

Those skilled in the art will be able to ascertain with no more than routine experimentation numerous equivalents to the specific processes and products described herein. Such equivalents  
20 are considered to be within the scope of the invention and are intended to be embraced by the following claims in which we claim:



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(ii) TITLE OF INVENTION: IN VITRO DIFFERENTIATION OF CD34+ PROGENITOR  
CELLS INTO T LYMPHOCYTES

(iii) NUMBER OF SEQUENCES: 3

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35 (A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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5

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## 20 (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## 30 (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

35 (A) ORGANISM: Macaca mulatta

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCCCGGGCG CTGCA

15

5 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Macaca mulatta

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTCCACAC GCTTGCACT

19

25 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Macaca mulatta

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGTTGGGGTCG GTGTCAGCCA CTCTCACCTC CC

32

**CLAIMS**

1. A method for *in vitro* T cell production, comprising:  
coculturing *in vitro* a monolayer of non-human primate thymic stromal cells with primate hematopoietic T cell progenitor cells.  
5
2. The method of claim 1, wherein the hematopoietic T cell progenitor cells are derived from a member of the species Homo sapiens.
3. The method of claim 1, wherein the hematopoietic T cell progenitor cells are derived from  
10 a member of the species Macaca mulatta.
4. The method of claim 1, wherein the thymic stromal cells are derived from a member of the species Macaca mulatta.
- 15 5. The method of claim 2, wherein the thymic stromal cells are derived from a member of the species Macaca mulatta.
6. The method of claim 3, wherein the thymic stromal cells are derived from a member of the species Macaca mulatta.  
20
7. The method of claim 1, further comprising:  
isolating the hematopoietic T cell progenitor cells or descendants thereof from the monolayer of thymic stromal cells prior to the differentiation of such cells into predominantly CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup> and CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup> cells.  
25
8. The method of claim 7, further comprising:  
culturing the isolated cells with a mitogenic expansion agent to stimulate mitogenic expansion of the isolated cells or descendants thereof;  
isolating the expanded population of cells; and  
30 coculturing the expanded population of cells with a monolayer of thymic stromal cells.
9. The method of claim 7, wherein the predominant cell type isolated is CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup> cells.

10. The method of claim 1, 2, 3, 4, 5 or 6, further comprising:  
isolating the hematopoietic T cell progenitor cells or descendants thereof from the monolayer of thymic stromal cells; and  
culturing the isolated cells with a mitogenic agent to stimulate expansion of the isolated  
5 cells.
11. The method of claim 10, wherein the mitogenic agent is nonmitotic feeder cells.
12. The method of claim 1, 2, 3, 4, 5 or 6, further comprising:  
10 coculturing *in vitro* the monolayer of thymic stromal cells and the hematopoietic T cell progenitor cells in the presence of an agent capable of genetically altering the hematopoietic T cell progenitor cells; and  
isolating at least one genetically altered hematopoietic T cell progenitor cell or descendant thereof.  
15
13. The method of claim 1, 2, 3, 4, 5 or 6, wherein the hematopoietic T cell progenitor cells are genetically altered T cell progenitor cells.
14. The method of claim 5, further comprising:  
20 isolating the hematopoietic T cell progenitor cells from the monolayer of thymic stromal cells; and  
coculturing the isolated cells with irradiated peripheral blood lymphocytes in the presence of interleukin-2 to stimulate expansion of the isolated cells.
- 25 15. A method for testing the effect of an agent on cells, comprising:  
coculturing *in vitro* a monolayer of non-human primate thymic stromal cells with primate hematopoietic T cell progenitor cells in the presence of the agent; and  
comparing differentiation or growth of the T cell progenitor cells, or descendants thereof, exposed to the agent to the differentiation or growth of control T cell progenitor cells or  
30 descendants thereof as a determination of the effect of the agent.
16. The method of claim 15, wherein the hematopoietic T cell progenitor cells are derived from a member of the species Homo sapiens.

17. The method of claim 15, wherein the hematopoietic T cell progenitor cells are derived from a member of the species Macaca mulatta.
18. The method of claim 15, wherein the thymic stromal cells are derived from the thymus of a member of the species Macaca mulatta.
19. The method of claim 18, wherein the hematopoietic T cell progenitor cells are derived from a member of the species Homo sapiens.
20. The method of claim 15, wherein the hematopoietic T cell progenitor cells are genetically altered T cell progenitor cells.
21. The method of claim 20, wherein the hematopoietic T cell progenitor cells are derived from a member of the species Homo sapiens.
22. The method of claim 20, wherein the hematopoietic T cell progenitor cells are derived from a member of the species Macaca mulatta.
23. The method of claim 20, wherein the thymic stromal cells are derived from a member of the species Macaca mulatta.
24. The method of claim 23, wherein the hematopoietic T cell progenitor cells are derived from a member of the species Homo sapiens.
25. A kit comprising:  
a container containing a cryopreserved non-human primate thymic stromal cell suspension; and  
instructions for coculture of the thymic stromal cells with primate hematopoietic T cell progenitor cells.
26. An article of manufacture comprising:  
a container containing a representative population of T cells derived by the method of claim 1.

27. An article of manufacture comprising:

a container containing a representative population of T cells derived by coculturing *in vitro* a monolayer of non-human primate thymic stromal cells with primate hematopoietic T cell progenitor cells;

5 isolating the hematopoietic T cell progenitor cells or descendants thereof from the monolayer of thymic stromal cells; and

culturing the isolated cells with a mitogenic agent to stimulate expansion of the isolated cells.



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/05607

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/02

US CL : 435/240.2, 240.21, 240.21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.21, 240.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, BIOSIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,085,985 (MAINO ET AL.) 04 February 1992, see entire document.	1-27

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Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 JUNE 1996

Date of mailing of the international search report

02 JUL 1996

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